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# INTRADERMAL IMMUNIZATION OF MONKEYS WITH ONE SET OF INJECTIONS OF POLIOMYELITIS VIRUS

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(Received for publication, October 10, 1929)

The problem of artificial immunization in poliomyelitis continues to hold attention because of the reappearance of epidemics of poliomyelitis in different parts of the world and the increasing knowledge of immunity to the class of so-called virus diseases, of which poliomyelitis is one example. That it is possible to produce an artificial active immunity in monkeys to the poliomyelitis virus by repeated sub-clinical, subcutaneous, and intradermal injections of the virus was shown originally by Flexner and Lewis in 1910 (2), later by Aycock and Kagan (3), and recently by Stewart and Rhoads (1). The general literature on the subject has been reviewed by the last named authors in their paper.

More recently still we have carried out a test on four *Macacus rhesus* monkeys in which repeated intradermal injections of a highly potent virus replaced the injections of the weaker M.A. and Aycock strains. When the less potent strains are employed, immunization usually takes place without clinical manifestations, but when the highly potent strain is used, the result is different:—The monkeys of our test became ataxic, tremulous, and excited about a week after the third intracutaneous injection, or, in other works, presented the appearances manifested by ordinarily inoculated monkeys a day or two before the onset of paralysis. Of the four monkeys showing the preparalytic symptoms, three recovered completely—that is, the symptoms aborted—while the fourth became typically paralyzed and succumbed to experimental poliomyelitis, the spinal cord and medulla revealing characteristic lesions. Flexner and Lewis described the occurrence of isolated instances of paralytic poliomyelitis in monkeys undergoing subclinical immunization, and Aycock and Kagan and Stewart and Rhoads

noted the same occurrence. The greater number of monkeys, when injected repeatedly with weak virus strains, become actively immune without exhibiting abortive symptoms.

These observations raise the question whether repeated minute inoculations of poliomyelitis virus do not act in certain instances to render the treated monkeys, not more resistant but more susceptible to subsequent injections into skin and subcutaneous tissue, such as would usually be ineffective. It seemed possible that a larger quantity of potent virus given intradermally at one injection might accomplish active immunization without inducing any symptoms whatever. The experiment now to be recorded constitutes a test of this second point.

## EXPERIMENTAL

Four *Macacus rhesus* monkeys were prepared by shaving the ventral surface from the clavicle to the pubis, and cleaning the skin carefully with alcohol. The material used was a 5 per cent physiological saline suspension of recently glycerolated spinal cord of the pooled, mixed virus strain. This strain is one of known constant activity; doses as small as 0.005 cc. of Berkefeld filtrate are uniformly infective when inoculated intracerebrally, and repeated small doses of the same strain given intradermally produce poliomyelitis. Inoculation was performed with a fine needle on a tuberculin syringe, and evenly spaced blebs of the material, about 0.5 cm. in diameter, were introduced into the skin, until the entire amount, 16 cc., had been given. Great care was observed to prevent the material from entering the subcutis, to obviate the possibility of too rapid diffusion of the virus. The animals were examined daily both for symptoms of poliomyelitis and for local reaction in the skin area treated. At no time were any abnormalities observed.

A month was allowed to elapse and the animals were bled for neutralization tests. This procedure was deemed advisable since Stewart and Rhoads (1) have shown that the power of serum to neutralize virus is a more delicate test of immunity than is the resistance of an animal to actual inoculation of the virus itself. Each serum obtained was set up in a volume of 0.99 cc. against 0.01 cc. of a Berkefeld N filtrate, prepared from a 5 per cent physiological saline solution suspension of fresh poliomyelitis spinal cord. The mixtures were placed in the incubator for two hours and in the icebox overnight. The following morning each was inoculated intracerebrally in a normal monkey.

The power of the serum to neutralize virus was compared with the resistance of the animals to actual introduction of infectious material into the central nervous system. The treated monkeys with a normal control were inoculated in the cisterna magna with 0.01 cc. of virus filtrate—about five minimal lethal doses of the same strain. Since intracerebral inoculation involves considerable trauma the cisternal route was selected as providing conditions more nearly resembling those in man when infection occurs (4).

## *Results*

The results of the experiments are summarized in the tables. The neutralizing power of the sera tested is shown in Table I. Neutralization was complete in every case, whereas in the control instance an

TABLE I

*Serum Neutralization*

| Monkey No. | Treatment | | | Symptoms | Neutralization | | Result |
|---|---|---|---|---|---|---|---|
| | Virus | Route | Amount | | Serum | Virus | |
| | | | *cc.* | | *cc.* | *cc.* | |
| 1 | M. V. | Intradermal | 15 | 0 | 0.99 | 0.01 | No symptoms |
| 2 | M. V. | Intradermal | 15 | 0 | 0.99 | 0.01 | No symptoms |
| 3 | M. V. | Intradermal | 15 | 0 | 0.99 | 0.01 | No symptoms |
| 4 | M. V. | Intradermal | 15 | 0 | 0.99 | 0.01 | No symptoms |
| 5 | — | Control | — | — | 0.99 | 0.01 | Typical poliomyelitis |

TABLE II

*Cistern Test*

| Monkey No. | Treatment | | | Symptoms | Inoculation | | Result |
|---|---|---|---|---|---|---|---|
| | Virus | Route | Amount | | Route | Dose | |
| | | | *cc.* | | | *cc.* | |
| 1 | M. V. | Intradermal | 15 | 0 | Died—intercurrent disease | | — |
| 2 | M. V. | Intradermal | 15 | 0 | Cistern | 0.01 | Typical poliomyelitis 10 days—recovered |
| 3 | M. V. | Intradermal | 15 | 0 | Cistern | 0.01 | No symptoms |
| 4 | M. V. | Intradermal | 15 | 0 | Cistern | 0.01 | No symptoms |
| 5 | — | Control | — | — | Cistern | 0.01 | Typical poliomyelitis 4 days—died |

equal amount of virus treated with normal serum led to typical poliomyelitis after a seven day incubation period. It therefore appears clear that the intradermal inoculation described produced a definite degree of immunity.

Table II shows the results of intracisternal inoculation of virus into

the monkeys that had received the immunizing treatments. As one animal of the series died of intercurrent disease, only three treated animals could be tested. Two remained free of all symptoms, while the third developed typical symptoms of poliomyelitis, became paralyzed in extremities and back, was prostrate for some time, but recovered, although marked residual paralysis persisted. The inoculated control animal, on the other hand, became paralyzed on the fourth day, prostrate on the fifth, and died on the sixth. The difference in the results of the tests in the treated and control animals is rendered more striking by the fact that the strain of virus used, when injected into normal monkeys, was almost invariably fatal within eight days.

## CONCLUSIONS

1. By means of a single large dose of poliomyelitis virus, distributed at a number of intradermal sites, active immunity has been produced in *Macacus rhesus* monkeys, as shown by skin neutralization and intracisternal tests.

2. In the small series (four animals) of monkeys so treated, neither abortive nor paralytic signs of experimental poliomyelitis appeared.

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# THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY

## VI. The Effect of Endothelial Blockade on the Storage and Distribution of Neoarsphenamine

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(Received for publication, September 4, 1929)

For two main reasons scientific workers have studied the fate of arsphenamine and of its various derivatives after parenteral introduction into the body. It is important to know the extent to which the drug is capable of penetrating into the various tissues and the duration of its retention if we are to form any opinion on the degree of its toxicity and its power of reaching parasites in remote places. Of equal importance, though of more theoretical nature, is the knowledge of what kinds of cells are concerned with the storage of the chemotherapeutic agent and what physical and chemical changes the drug undergoes in the body. These questions must be answered before one can hope to throw any light on the mechanism of the chemotherapeutic effect.

A brief review of the more recent literature shows that arsenicals of the arsenobenzene type upon intravenous injection leave the blood stream largely within a few minutes to be taken up subsequently—in decreasing order of amount—by the liver, spleen, lungs, bone marrow, kidneys, brain and sceletal muscles (Voegtlin and Thompson (1), Kolls and Youmans (2), Clausen and Jeans (3), Minot (4), Bulmer (5), Wechselmann, Lockemann and Ulrich (6)). The excretion of the drug with the feces and urine apparently sets in very rapidly (2 to 5 minutes after the injection) and continues for the following 24 to 48 hours, (Beeson and Albrecht (7), Clausen and Jeans, Underhill and Davis (8)) after which time the process of elimination is practically completed. According to several authors traces of arsenic may be detected in the excreta up to much later periods and at the end of 48 hours more than 50% of the injected dose is said to remain quantitatively unaccounted for. As a matter of fact, Voegtlin and Thompson established a definite relation between the velocity of excretion and the chemotherapeutic

efficiency, which indicated to them that "temporary retention of the arsenic is unquestionably an essential requirement for good chemotherapeutic action."

Dale (9) has presented an impressive array of facts which would seem to throw some doubt on the validity of the earlier conception of Ehrlich according to which the chemotherapeutic effect of a given substance is determined by the relation between parasitotropism and organotropism—a strong affinity for the protoplasm of the parasite and a weak affinity for that of the cells of the host being highly essential for good chemotherapeutic action. The difficulties encountered in proving by chemical means a differential affinity for parasite and tissue cells together with other reasons have since induced an increasing number of investigators to regard some participation of the host as indispensable to the accomplishment of the chemotherapeutic effect. More serious objections to the Ehrlich axiom arose from the fact that removal of the spleen or blockade of the reticulo-endothelial system resulted in a marked impairment or, in some cases, complete abolition of the chemotherapeutic action of arsphenamine and other chemotherapeutic agents (Kritschewski and Meersohn (10), Feldt and Schott (11), Jungeblut (12)). The intervention of a specialized system of cells was thus, for the first time, proved to be indispensable for the accomplishment of the full chemotherapeutic effect. More significance was lent to these empirical findings, when v. Jancso (13) and Imenez de Asua and Kuhn (14) succeeded, independently, in demonstrating by special histiochemical staining methods the actual presence of the drug in the histiocytes of the liver and in other cells belonging to the reticulo-endothelial system.

In attempting to explain the deleterious effect of blockade or splenectomy on the chemotherapeutic efficiency of arsphenamine, different interpretations were offered. There are of course those who believe that the destruction of the parasite by the drug is due to the combined action of chemical agencies and immune bodies (Kolle and others). Extremists deny any interaction between parasite and drug and attribute the sterilization effect solely to enhanced cellular activity (Feldt). Among other factors we (Jungeblut) discussed particularly the possibility that the transformation of the compound inactive *in vitro* into the highly parasiticidal drug *in vivo* may take place in the reticulo-endothelial cells. It seems to us, that evidence since produced, would not tend to corroborate this hypothesis, at least not in its original simple form, and we are now inclined to maintain a viewpoint, which approaches the opinion of Kritschewski (15), voiced after revision of his first attempt of explanation. We believe that rapid initial storage and gradual subsequent release of arsphenamine is an indispensable prerequisite if the drug is to display an optimum chemotherapeutic effect in the body. It is highly probable that a similar mechanism comes into play with other drugs, such as Bayer 205 (Germanin) and quinine (Sei (16), Roehl (17), Freund (18), Mayer and Zeiss (19), Giemsa (20), and Tchapkewitch (21)). Whether the rôle of the reticulo-endothelial system during this phase is simply one of providing a depot and store for the chemotherapeutic agent, or whether important chemical changes are mediated by those cells is, at present, largely a matter of speculation, at least as far as the

second possibility is concerned. The first question, however, is readily open to direct experimentation.

The present paper deals with an attempt to determine by quantitative chemical analysis the rate of disappearance of neoarsphenamine from the circulation after intravenous injection in blocked and normal animals and to compare the arsenic content of the liver and spleen within the two groups.

## EXPERIMENTAL WORK

In order to secure more complete information, the work was planned to include observations with three different species of animals, *i.e.* guinea pigs, rabbits, and mice. Guinea pigs and rabbits were used for measurements of arsphenamine in the blood, while determinations of the arsenic content of the liver and spleen were carried out with mice. The method of blockade followed closely the procedure employed in former experiments. Inasmuch as the Abelin reaction (22) has been found reliable by previous workers, we have used this test in quantitative form for the determination of neoarsphenamine in samples of blood. The arsenic determinations in the tissues were carried out with the Gutzeit method, as modified by Sanger and Black (23). The results obtained with the three different series are reported under separate headings as follows:

*Series I.*—A total of sixteen guinea pigs weighing from 300 to 400 grams were used. Eight of these were normal controls, the remaining eight received by intravenous injection 1.5 cc. of a 1:5 dilution of India ink. All animals were injected intravenously with 1 cc. of a 1:70 dilution of neoarsphenamine, the interval between blocking injection and injection of the drug being approximately 24 hours. One half of the animals were bled to death five minutes after the injection of neoarsphenamine, the other half at the end of twenty minutes. The blood was allowed to clot and 1 cc. of clear serum was used from each animal as sample in carrying out the Abelin test. We followed the original method described by Abelin and obtained rather sharp and well defined colour reactions for a range of neoarsphenamine dilutions varying from 1:1000 to 1:50,000. Although the reaction as originally described by Abelin was only a qualitative test, it may be used for quantitative determination with a fair degree of accuracy by comparing the colour shades obtained with the test material with those of a freshly prepared scale of neoarsphenamine solutions of known concentrations. The results obtained in this series appear in Table I.

In spite of individual variations from animal to animal, which undoubtedly depend to a large extent upon the varying total blood supply, the figures show very clearly a much slower absorption of neoarsphenamine from the blood stream of blocked animals as compared

with normal controls. Taking the average as a basis of comparison it would appear that the ratio is approximately 1:2. Assuming the average total volume of plasma of these guinea pigs to be about 7 cc., the figures mean that five minutes after the injection the blocked animal still retains in the blood about one half of the total dose of neoarsphenamine injected while the concentration of the drug in the blood of the normal animal is only one fourth. After the twenty

TABLE I

*Determinations of Neoarsphenamine in the Blood of Blocked and Normal Guinea Pigs**

(*Abelin Test*)

| Blocked** Guinea Pigs | Interval *** | | Normal Guinea Pigs | Interval*** | |
|---|---|---|---|---|---|
| | 5 min. | 20 min. | | 5 min. | 20 min. |
| | *mg. of Neo per 1 cc. of serum* | | | *mg. of Neo per 1 cc. of serum* | |
| 1 | 1.18 | | 9 | .59 | |
| 2 | | .47 | 10 | | .39 |
| 3 | 1.3 | | 11 | .36 | |
| 4 | | 1.0 | 12 | | .33 |
| 5 | .40 | | 13 | .40 | |
| 6 | | .31 | 14 | | .20 |
| 7 | 1.2 | | 15 | .80 | |
| 8 | | .72 | 16 | | .58 |
| Average..... | 1.02 | .63 | Average..... | .54 | .38 |

* All animals received intravenously 1 cc. of a 1:70 dilution of neoarsphenamine. Total dose injected: 14.28 mg. Neo.

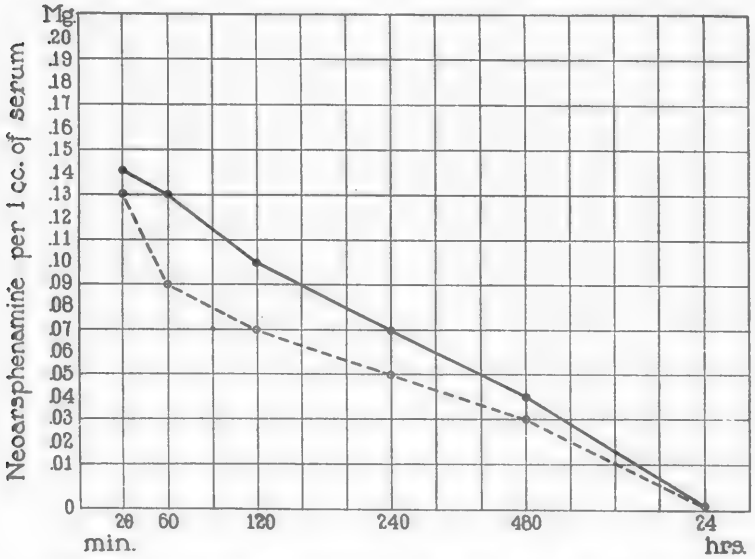
** These animals received intravenously 1.5 cc. of a 1:5 dilution of India ink 24 hours before injection of the drug.

*** Time elapsed between injection of the drug and obtaining of sample.

minute interval the figures are reduced to approximately one fourth of the total dose for the blocked animal against one eighth for the controls. Those figures are of course not absolute values, the object of the experiment lying chiefly in the relative comparison between experimental and control animals. It will also have to be remembered, that the Abelin reaction is no test for arsenic, but indicates merely the presence of the unchanged drug, *i.e.* the whole organic complex.

In order to supplement the data obtained in the first series with information regarding the rate of absorption during longer periods of time in the same animal, we have followed the disappearance of the drug from the circulation of rabbits in a second series of experiments.

*Series II.*—A total of four rabbits, two of them blocked by a preceding intravenous injection of India ink (5 cc. of a 1:5 dilution), two serving as normal




GRAPH 1. Elimination of neoarsphenamine from the blood of blocked and normal rabbits. Total dose injected: 30 mg. neoarsphenamine. ——— Blocked animal (combined results of two different tests). ---------- Normal animal (combined results of two different tests).

controls, received each 3 cc. of a 1:100 dilution of neoarsphenamine into the ear vein. Blood samples (5 cc.) were obtained by heart puncture from each rabbit at various intervals, covering periods of from twenty minutes up to 24 hours since the injection of the drug. The Abelin test was carried out with 1 cc. of clear serum from each sample as described before.

Graph 1 shows the different curves of elimination. Although the differences between blocked animals and controls are not as marked as in series I, they show a distinctly slower elimination of the drug

from the circulation of the blocked rabbit. Our observations in normal guinea pigs and rabbits show otherwise a somewhat more abrupt fall in concentration of the circulating arsenical than the figures, which Schreus and Hollaender (24) established, would indicate for humans.

TABLE II

*Determinations of Arsenic in the Livers of Blocked and Normal Mice Following the Injection of Neoarsphenamine**

(*Gutzeit Method*)

| | Blocked Mice** | | | Normal Mice | | |
|---|---|---|---|---|---|---|
| Interval | Weight of liver | Neo*** per 0.5 g. of tissue | Neo*** per whole organ | Weight of liver | Neo*** per 0.5 g. of tissue | Neo*** per whole organ |
| *minutes* | *g.* | *mg.* | *mg.* | *g.* | *mg.* | *mg.* |
| 5 | 1.80 | .23 | .828 | 1.20 | .33 | .792 |
| " | 1.13 | .15 | .339 | 1.02 | .50 | 1.02 |
| 20 | 1.15 | .21 | .483 | 1.07 | .43 | .920 |
| " | 1.37 | .25 | .685 | 0.72 | .60 | .864 |
| 30 | 3.15 | .20 | 1.26 | 1.50 | .50 | 1.50 |
| " | 0.90 | .10 | .18 | 1.60 | .30 | .96 |
| 60 | 1.12 | .25 | .56 | 1.49 | .40 | 1.192 |
| " | 1.38 | .10 | .276 | 1.25 | .30 | .75 |
| 120 | 1.14 | trace | — | 1.12 | .05 | .112 |
| " | 1.00 | .05 | .10 | 1.40 | trace | — |

* All animals received i.v. 1 cc. of a 1:250 dilution of noarsphenamine. Total dose injected: 4 mg. Neo.

** These animals received i.v. 1 cc. of a 1:15 dilution of India ink 24 hours before injection of the drug.

*** The neoarsphenamine figures are computed by multiplying the arsenic values by five. The brand of neoarsphenamine used contained about 20% As.

In a third series of experiments the arsenic content of the liver of blocked and normal mice was determined, following the intravenous injection of neoarsphenamine.

*Series III.*—A total of 20 animals, which weighed from 20 to 25 grams, received a uniform dose of 1 cc. of a 1:250 dilution of the drug. One half of them were

normal controls, the other half had been blocked by a preceding intravenous injection of India ink (1 cc. of a 1:15 dilution). Duplicate animals in either group were killed at five, twenty, thirty, sixty, and 120 minutes, respectively, after injection of the drug, and the liver, including the gallbladder, was carefully removed *in toto*. After accurately determining the weight of the organ, an aliquot portion of the organ (0.5 g.) was finely ground in the mortar. The organic matter in the tissue being destroyed by the usual treatment with boiling $H_2SO_4$ and $HNO_3$, the arsenic content in the residue was determined by Sanger and Black's modification of the Gutzeit method. The computed neoarsphenamine figures are tabulated in Table II.

As may be gleaned from Table II the figures show on an average approximately half as much arsenic in the blocked liver as in the normal organ, comparing at similar intervals gram for gram of tissue. Limiting our observations to the first hour we found in the whole normal liver approximately from one fourth to one fifth of the total amount of arsenic injected while the fraction of arsenic present in the whole blocked liver has varied from one tenth or less to one fifth of the total dose. Although the number of determinations is small and the individual figures show considerable variation, it would seem that the maximum concentration of the drug in the liver is established during the course of the first hour, after which time there is a rather rapid decline in the amounts of arsenic recovered. Several attempts to measure the arsenic content in the spleen have yielded amounts too small to be measured accurately with our technique. Our results have thus failed to bear out Schlossberger's (25) expectations.

## DISCUSSION

Our findings are in the main in agreement with the experience of many authors that blockade of the reticulo-endothelial system leads to a slower elimination of "storable" colloids from the blood stream and consequently to a less complete impregnation of the reticulo-endothelial cells with these substances (for a review of the literature see Jungeblut (26)). As far as the present work is concerned it appears that the absorption of neoarsphenamine from the blood stream after intravenous injection of the drug is materially retarded in guinea pigs which received a single blocking injection of India ink 24 hours before, as compared with normal controls. This difference is more apparent at the earlier intervals following the injection of the drug

than at later stages. The elimination curves in the rabbit show a less marked, but nevertheless very regular and characteristic lag of the blocked animal against the normal control. As far as the final residue in the circulation after 24 hours is concerned, we have not been able to detect any differences with either group of animals.

The above results harmonize well with the information obtained from arsenic determinations in the tissues of blocked and normal mice after the intravenous introduction of the same drug. The facts here indicate in brief that the concentration of neoarsphenamine in the blocked liver is less than one half of the amount present in the normal organ, comparing at similar intervals during the first hour's observation gram for gram of tissue. Blockade of the reticulo-endothelial system, in other words, alters profoundly the storage and distribution of neoarsphenamine, causing shortly after injection of the drug more neoarsphenamine to remain in the circulation and less to accumulate in the tissues.

To what extent the observations made in this paper serve to elucidate the mechanism of chemotherapeutic action, is a question which at present can be answered only tentatively. Those who argue that the arsphenamine in the tissues is negligible for the chemotherapeutic action and accordingly credit the circulating arsphenamine with the sterilization effect (v. Jancso, Schlossberger, Wechselmann, Lockemann and Ulrich) will find it difficult to reconcile such an hypothesis with the fact that blocked animals, whose blood actually contains larger amounts of the drug, die from the experimental infection while normal controls survive with much smaller concentrations of arsphenamine in the circulation. On the other hand, if we are to attribute causal significance to the process of storage by the tissue cells for the chemotherapeutic efficiency of the drug, we are baffled by the recent paradox observations of v. Jancso (27). While, according to this author, among the arsenicals of the arsenobenzene type, those which possess a high degree of tissue affinity, also exhibit the highest trypanocidal action, there is no parallelism demonstrable between reticulo-endotheliotropism and the chemotherapeutic efficiency as regards the action of the various derivatives on spirochaete infections. Finally, the peculiar intervention of the intact reticulo-endothelial system is apparently not restricted to the arsenicals, but governs as

well the pharmacodynamics of such totally different substances as Bayer 205 (Germanin).

While evidently the problem is still far from a perfect solution, we feel inclined to attribute the impairment of the chemotherapeutic efficiency of the arsphenamines by blockade of the reticulo-endothelial system in general to an interference with the storage of the drug by the histiocytic cells. Following the indicated trend of thought it would seem that the blocked animal is distinctly handicapped in its fight against the experimentally produced infections by the initial lag of the resorptive power of the liver and possibly by a disturbance of the regulative function of the organ for the subsequent release of the drug, probably in some modified form. The composite picture of the fate of the drug in the blocked animal is necessarily incomplete without knowing the effect of blockade on the rate of excretion through the feces and urine; we therefore prefer to reserve final judgment until such information is available. Further work will also have to show whether *increased* activity of the reticulo-endothelial cells, induced by suitable stimulation, might not result in a *higher chemotherapeutic effect,* an expectation which would not seem to be altogether ungrounded in view of the fact that quite recently Roskin and Romanowa (28) have been able to show that irradiation of the experimental animal with ultraviolet light will enhance the chemotherapeutic effect.

## CONCLUSIONS

1. Blockade of the reticulo-endothelial system by means of a single injection of India ink caused a marked retention of neoarsphenamine in the blood of guinea pigs during the first twenty minutes of observation after intravenous injection, as contrasted with the rapid disappearance of the drug from the blood of normal controls.

2. Rabbits blocked by a single dose of India ink showed a slower elimination of the drug from the circulation following the first few hours after intravenous injection than corresponding controls.

3. The arsenic content of the liver of mice, which received neoarsphenamine intravenously after a preceding blocking injection with India ink, was appreciably lower than the arsenic content of the normal organ under similar experimental conditions.

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# THE RÔLE OF THE RETICULO-ENDOTHELIAL SYSTEM IN IMMUNITY

## VII. The Effect of Local and Systemic Blockade on the Toxic Action of Heterophile (Forssman) Sera in Guinea Pigs

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### INTRODUCTION

During the past few years the relations between anaphylactic phenomena and the function of the reticulo-endothelial system have received a large share of scientific interest. In a critical review we (Jungeblut (1)) have recently surveyed the vast amount of information which has accumulated from work in this field. While all efforts, undertaken with the view point of employing the blockade method in the study of anaphylaxis for the prevention of systemic anaphylactic shock, have yielded but uncertain or contradictory results, more significant observations have been made by Klinge (2) who succeeded, in preventing completely the development of the local Arthus phenomenon in sensitized rabbits, provided the antigen was reintroduced into a tissue area, which had been locally blocked by a suitable dose of trypan blue. The toxic action of heterophile antisera in animals belonging to the 'guinea pig type,' which bears a striking resemblance to the symptomatology and pathology of allergic reactions, is characterized by the occurrence of severe tissue lesions, more extensive than what is generally observed in typical protein hypersensitiveness. It has therefore seemed worthwhile to us to investigate, to what extent cells of the reticulo-endothelial system are involved in the mechanism of this reaction.

As far as we are aware, there is only one observation of Forssman and Skoog (3) recorded in the literature, which deals with the present subject. These authors have reported, that injections of India ink,

while protecting against the so-called 'carotal symptom complex,' have no influence on the occurrence of the inverse anaphylactic reaction.

## EXPERIMENTAL WORK

In the following experiments we studied the effect of systemic blockade upon the development of systemic shock in the guinea pig following the injection of Forssman sera. At the same time we investigated the influence of local blockade on the occurrence of the local skin reaction in the animal.

For the first part of the experiments both blocking agent and heterophile serum were introduced by the intravenous route, while in the second part the serum was injected intracutaneously into areas of skin, which had previously been infiltrated with the blocking substance. We have used India ink as well as trypan blue for purpose of blockade. The work was planned so as to include observations after different intervals between the establishment of blockade and the injection of the toxic serum. The latter was obtained by immunization of rabbits with an emulsion of guinea pig kidney cells; for part of the work we have also employed an anti-sheep cell hemolytic serum.

*Series I.*—In the first series a group of six guinea pits were injected intravenously with 2 cc. of a 1:5 dilution of India ink and received at various intervals later a dose of 1 cc. of an anti-sheep cell rabbit serum by the same route. Three control animals were injected with the serum alone. This particular serum, upon careful preliminary titration for toxicity, had yielded a minimum lethal dose of 0.75 cc.* The results are tabulated in Table I.

*Series II.*—We have compared in this series the respective effect of India ink and trypan blue as blocking agents under otherwise similar experimental conditions. A total of seven guinea pigs were blocked with 2 cc. of a 1:5 dilution of India ink and an equal number of animals were prepared with 2 cc. of a 1% solution of trypan blue. All fourteen animals were injected intravenously at various intervals since establishment of blockade with 0.75 cc. of an anti-guinea pig kidney cell rabbit serum, the minimum lethal dose of which had been found before to be 0.5 cc. For purpose of control we have included in this series again three normal animals which received only the serum. Table II shows the results obtained.

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* We have designated as minimum lethal dose the smallest amount of serum, which upon intravenous injection into a guinea pig (weight from 250–350 g.) causes the death of the animal within five minutes. Somewhat smaller amounts have generally killed the animal within from ten to thirty minutes or on the following day.

A study of the results obtained with the two experimental series reveals that blockade of the reticulo-endothelial system has in the majority of the cases resulted in a prolongation of the time of survival of the guinea pigs, without however protecting the animals definitely against the lethal action of the toxic serum. This modification of shock was only fully apparent when several minutes had elapsed between establishment of blockade and the injection of the heterophile serum. As a rule it was noticeable up to a 24 hour interval. We have not

TABLE I

*Effect of Systemic Blockade with India Ink on the Systemic Shock Following Intravenous Injection of Heterophile Serum† in the Guinea Pig*

| Guinea pig No. | Injections of India ink | Injection of serum | Interval min. | Results* |
|---|---|---|---|---|
| 1 | 2 cc. of 1:5 dilution i.v. | 1 cc. i.v. | 5 | Died 3 min. |
| 2 | " | " | 30 | Died 17 min. |
| 3 | " | " | 60 | Died 20 min. |
| 4 | " | " | 90 | Died 26 min. |
| 5 | " | " | 120 | Died 1½ hours |
| 6 | " | " | 24 hours | Died 2 min. |
| 7 | — | " | — | Died 3 min. |
| 8 | — | " | — | Died 4 min. |
| 9 | — | " | — | Died 3 min. |

† Anti-sheep red cells rabbit serum.

* The symptoms before death resembled very closely those of typical anaphylactic shock. The autopsy findings showed inflation of the lungs and multiple hemorrhages in the kidneys, liver, spleen and lungs.

found either of the two blocking agents—India ink or trypan blue—superior to the other. We infer from the fact that the simultaneous injection of the blocking agent and the immune serum was followed by typical shock, that neither the ink nor the dye has exerted any direct detoxifying action on the serum.

In view of the uncertain results obtained with systemic blockade applied to the study of systemic shock, we have carried out further experiments in which the influence of local blockade on the development of the local tissue reaction was studied.

*Series III.*—In these experiments we have infiltrated a local area of skin (shaved abdominal region) with suitable doses of either India ink (1 cc. of a 1:20 dilution) or trypan blue (0.5 cc. to 1 cc. of a 1% solution). The intracutaneous injection of the serum was carried out at various intervals later; the serum was injected not only into the maximally blocked area but also into the adjoining areas of partially blocked skin and likewise into normal skin areas of the same animal, into

TABLE II

*Effect of Systemic Blockade with Either India Ink or Trypan Blue on the Systemic Shock Following Intravenous Injection of Heterophile Immune Serum† in the Guinea Pig*

| Guinea pig No. | Blocking injections | Injection of serum | Interval min. | Results* |
|---|---|---|---|---|
| | *i.v.* | *i.v.* | | |
| 1 | India ink. 2 cc. 1:5 dil. | 0.75 cc. | none | Died 2 min. |
| 2 | " | " | 10 | Died 2 min. |
| 3 | " | " | 30 | Died 4 min. |
| 4 | " | " | 60 | Died 10 min. |
| 5 | " | " | 120 | Died 8 min. |
| 6 | " | " | 180 | Died 12 min. |
| 7 | " | " | 24 hours | Died 11 min. |
| 8 | Trypan blue 2 cc. 1% sol. | " | none | Died 1½ min. |
| 9 | " | " | 10 | Died 2 min. |
| 10 | " | " | 30 | Died 5 min. |
| 11 | " | " | 60 | Died 10 min. |
| 12 | " | " | 120 | Died 9 min. |
| 13 | " | " | 180 | Lived |
| 14 | " | " | 24 hours | Died 14 min. |
| 15 | — | " | — | Died 2 min. |
| 16 | — | " | — | Died 5 min. |
| 17 | — | " | — | Died 3 min. |

† Anti-guinea pig kidney cells rabbit serum.

* Symptoms and autopsy findings were as described in Table I.

which the blocking agent had not diffused. The latter served as controls. The serum used for these experiments was the same Forssman serum employed in the second series. A minimum amount of .2 cc. of a 1:5 dilution upon titration on the normal guinea pig skin caused a severe local reaction, consisting of hemorrhagic inflammation on the first day to be followed by distinct necrosis on the second or third day. We have employed this dose as test dose in our work.

A total of six guinea pigs were locally blocked with trypan blue as indicated and another six animals were prepared in a similar manner with India ink. The serum was injected immediately, and then at intervals ranging from ten minutes to 24 hours. The tissue reactions following the intracutaneous injections of the Forssman serum were read two hours after completion of the injection and observations were kept up for the next three days. The results, based on the 48 hour reading, appear in Table III.

TABLE III

*Effect of Local Blockade on the Development of the Local Skin Reaction Following Intracutaneous Injection of Heterophile Serum**

| Local blockade with trypan blue (1 cc. of a 1% solution subcut.) | | | | | Local blockade with India ink (1 cc. of a 1:20 dil. subcut.) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Reactions in | | | | | Reactions in | | |
| Guinea pig No. | Interval min. | maximally blocked area | partially blocked area | normal control area | Guinea pig No. | Interval min. | maximally blocked area | partially blocked area | normal control area |
| 1 | none | 3+ | 2+ | 3+ | 7 | none | 3+ | 3+ | 3+ |
| 2 | 10 | − | 2+ | 3+ | 8 | 10 | 3+ | 3+ | 3+ |
| 3 | 30 | ± | 2+ | 3+ | 9 | 30 | 3+ | 2+ | 3+ |
| 4 | 60 | − | 1+ | 3+ | 10 | 60 | 3+ | 3+ | 3+ |
| 5 | 180 | + | 2+ | 3+ | 11 | 180 | 2+ | 3+ | 3+ |
| 6 | 24 hours | ± | ± | 4+ | 12 | 24 hours | 3+ | 2+ | 3+ |

* An anti-guinea pig kidney cell serum was used (.2 cc. of 1:5 dil.). The results are based on a 48 hour reading.

Nomenclature: 4+ = very severe reaction with extensive necrosis, 1 × 1 cm.
3+ = severe reaction with necrosis, ½ × ½ cm.
2+ = moderate reaction with slight necrosis, ½ × ½ cm.
1+ = slight reaction, no necrosis, ½ × ½ cm.
± = very slight reaction, no necrosis, less than ½ × ½ cm.
− = no reaction.

As can be seen from Table III, there were either none or at the most extremely faint reactions in the trypan blue-blocked areas, during an interval up to 24 hours after establishment of the blockade. The adjoining areas of partially blocked skin likewise did not respond as much to the toxic action of the serum as the control sites in the normal skin which showed fully developed reactions. On the other hand, the areas blocked with India ink showed lesions fully as severe as the

control sites. The different effect of the two blocking agents, yielding opposite results, was as striking in the positive as in the negative case.*

## DISCUSSION

The fact that it is not possible by systemic blockade with either India ink or trypan blue to prevent the occurrence of fatal systemic shock following intravenous injection of heterophile immune serum in the guinea pig, is not surprising if we keep in mind the peculiar localization of the heterophile antigen *in vivo*. Forssman (4) considers the kidney as the chief source of the antigen, while according to the same author the liver and spleen contain much smaller amounts. Since the elements of the reticulo-endothelial system are primarily found in the liver and spleen and are absent in the kidney, it would seem that the vital dye has blocked precisely those cells which do not contain Forssman's antigen, leaving free access for the serum to the store of antigen in the kidneys.

In the case of the complete inhibition of the local skin reaction following intracutaneous injection of the heterophile immune serum into areas of skin, locally blocked with trypan blue, it is difficult to reason along analogous lines. Very little is known about the quantities of heterophile antigen present in the skin and subcutaneous tissue, although the severe reactions induced by the serum in the normal skin would, *a priori*, indicate a rich content of antigen in that structure. Details of distribution of the antigen amongst the different types of cells in the skin and subcutaneous tissue have, to our knowledge, never been investigated (5). Since the dye is taken up selectively by the clasmatocytes (histiocytes), and since trypan blue-blocked areas are insusceptible to the toxic action of the serum, it may not be inappropriate to suspect that those cells harbour the antigen. However, it is naturally impossible to make any definite statements on that point without careful histological examination of the particular areas. All the experiment proves is the fact that local blockade has interfered in

* We have also carried out a few experiments in which either the mouse or the dog was used as the test animal. While the mouse proved insusceptible to large doses of heterophile serum by either the intravenous or intracutaneous route, inhibition of the local skin reaction by local blockade in the dog has not been very clear cut. Our observations are too few, however, to warrant detailed discussion.

some way with the contact between the heterophile antibody in the immune serum and the corresponding antigen in the tissues. As regards the difference between the effect of trypan blue and India ink—the latter being incapable of preventing the full development of the local lesion—we are unable to offer any satisfactory explanation. It is interesting to note, that Klinge has also been unsuccessful in preventing the Arthus phenomenon in sensitized rabbits, when he used India ink as blocking agent, although the experimental conditions are not directly comparable. There is a possibility that the much greater diffusibility of the dye and perhaps its more profound injury to the cell protoplasma may endow it with its peculiar virtues.

## CONCLUSIONS

1. Guinea pigs blocked by intravenous injection of either India ink or trypan blue succumbed with fatal shock after the intravenous injection of heterophile immune serum, although the time of death was somewhat delayed as compared with normal controls.

2. Local blockade of the skin of guinea pigs with trypan blue inhibited completely the development of the characteristic skin reaction following intracutaneous injection of Forssman serum within the blocked area.

3. Infiltration of the skin of guinea pigs with India ink afforded no protection against the full development of the toxic skin reaction.

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# THE EFFECT OF THE X-RAY ON THE NODULES OF VERRUGA PERUANA

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Plate 1

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In a recent paper from this laboratory[1] it was shown that convalescent serum, taken from monkeys which had recovered from experimental verruga, has two evident effects on the disease as produced in monkeys by the inoculation of cultures of *Bartonella bacilliformis*.

The convalescent serum inhibits the development of verrugous nodules, that is, delays the appearance or slows-up the growth, but it does not prevent their ultimate appearance and their reaching considerable size. On the other hand, it effectively prevents the proliferation and persistence of *Bartonella bacilliformis* in the blood stream. It is hoped that the treatment of cases of Carrion's disease in man may be made more effective through the employment of the blood serum of convalescents from verruga and from Carrion's disease itself.

The curative influence of the X-ray on various skin lesions in man has suggested experiments to ascertain whether the rays, applied therapeutically, would affect the evolution of verruga nodules in monkeys receiving skin inoculations of *Bartonella bacilliformis*. The pathology of the lesions indicated that this was probable. In histological structure the verruga nodule[2,3,4] is a circumscribed collection of proliferating endothelial cells, arranged in groups and masses along blood and lymph spaces, in a manner resembling an endothelioma. Ordinary inflammatory changes such as exudation of polynuclears and round cells occur in mild degree only. Furthermore, the verruga nodules in spite of their definitely proliferative nature tend to regression ultimately. In this they differ essentially, of course, from the endotheliomata. The time period during which evolution and regression take place can be gauged quite accurately.

With these facts in mind, we undertook two sets of experiments.

In one we wished to observe the effect of a single application of X-ray on the nodules after they had just started to develop in monkeys, and in the other, to see whether the skin of a normal monkey which had received one application of X-ray would fail to develop verruga nodules on later inoculation.

### *Method*

After the monkey had been shaved it was stretched on a board and the chest and abdomen were covered with a lead-rubber mat 1/16 inch thick. The mat was so placed that the nodule to be treated was in the center of a hole in it 3 cm. square. It follows that an area of 9 square cm. in all was exposed to the X-ray, having at its center the nodule to be treated.

An attempt was made to find and use an X-ray dosage sufficient to produce only an erythema of the skin. Four, six and eight minute exposures at a distance of six inches, with a three inch spark gap and 10 M.A. were tested. It was found that sometimes a six or an eight minute exposure produced desquamation and a serous discharge from the area X-rayed, but in the type experiments reported here only erythema occurred.

### *Treatment of Growing Nodules*

*Macacus rhesus* X-1 was intradermally inoculated with living cultures of *Bartonella bacilliformis*, strain P5, in four places, two on the left side and two on the right side of the abdomen. An inoculation was also made between them into a scarified area on the midline. On the sixth day, nodules 3 to 4 mm. in diameter were distinctly visible and palpable at all the sites of intradermal inoculation (Fig. 1). The following day the two nodules on the left side were treated with X-ray, the left upper nodules for six minutes and the left lower nodule for only four minutes. The nodules on the right side and the scarified area were not exposed to the X-ray, serving as controls. These nodules on the right side progressed steadily; 26 days after inoculation the upper one was 12 mm. and the lower 20 mm. in diameter (Fig. 2). Seventy days after inoculation they had completely regressed and only small scars remained. The nodules on the left side, however, during this period, when those on the right were flourishing, remained stationary in size for about 14 days, and on the 46th day after inoculation had entirely disappeared.

*Macacus rhesus* X-2 was injected intradermally in six places on the abdominal wall, 3 on each side, with cultures of *Bartonella bacilliformis*, strain 2. A scarified area on the midline was also inoculated. Ten days later each intradermal site of inoculation showed a nodule 4 mm. in diameter (Fig. 3). The next day the nodules on the left side were treated with X-ray, 8 minutes for the upper nodule, 6 minutes for the middle nodule and 4 minutes for the lower nodule. The nodules on the right side and the scarified area were not treated and served as controls. On this,

the untreated side, the nodules were 15 mm. in diameter 34 days after inoculation (Fig. 4), and had completely regressed 63 days after inoculation. The nodules on the left, the treated side, remained stationary for about 10 days after treatment, with the surrounding skin slightly hyperemic and edematous. They then gradually regressed and disappeared (Fig. 4).

### *Treatment of the Skin Prior to Inoculation*

In *Macacus rhesus* X-5 the shaven skin of the anterior surface on the left side was exposed to the X-ray for 6 minutes, while the right side remained untreated and served as control. Then, 4 days later, two intradermal inoculations of cultures of *Bartonella bacilliformis*, strain 4, were made on each side of the animal; i.e., two on the treated and two on the untreated side. The nodules on the right, the untreated side, showed the usual progress. On the 46th day after inoculation they were two large pedunculated masses, each 20 mm. in diameter (Fig. 6), and 42 days later they had entirely disappeared. On the left, or treated side, there was marked retardation of the development of the nodules. Eight days after inoculation, they were just palpable and much smaller than those on the right which were red, prominent and 5 mm. across. On the 23rd day the nodules on the right were 10 mm. and 5 mm. respectively, and those on the left had entirely disappeared.

## SUMMARY AND CONCLUSIONS

The supposition that X-ray would affect the developing and the developed verruga nodules experimentally induced in the monkey, has proved correct. The experiments show that the early verruga nodules when exposed to a single, properly graduated dose of X-ray producing merely erythema of the skin, are inhibited in their evolution. Moreover, the skin of *Macacus rhesus* monkeys is modified by a single erythema dose of X-ray in such a way that infection of it with *Bartonella bacilliformis* is rendered more difficult.

These results are sufficiently striking to warrant the trial of X-ray in suitably guarded doses in the treatment of verruga nodules in man. Should the employment of convalescent serum influence the course of Carrion's disease favorably and the use of X-ray bring about a more certain and rapid devolution of verruga nodules in man, two practically applicable therapeutic measures will have been provided for the treatment of the two forms of the human disease.

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## EXPLANATION OF PLATE 1

FIG. 1. *Macacus rhesus* X-1, showing condition of verruga nodules 7 days after inoculation, before treatment with X-ray.

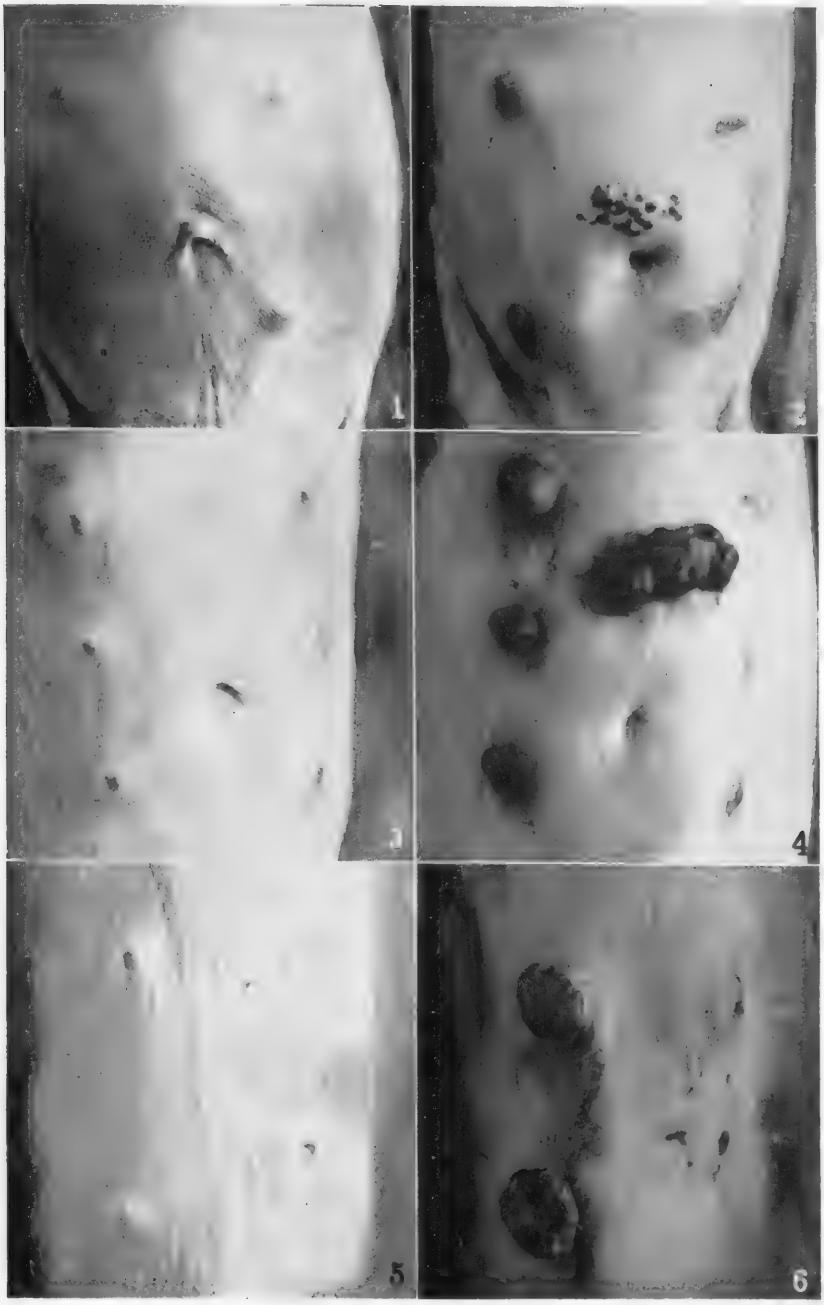
FIG. 2. Same animal 21 days after X-ray treatment. The two nodules on the left side are considerably smaller than the two untreated nodules on the right. Scarified area in the center is untreated.

FIG. 3. *Macacus rhesus* X-2. The six nodules 11 days after inoculation and before X-ray treatment.

FIG. 4. The same animal 23 days after X-ray treatment. The three nodules on the right side of the animal and the scarified area are strongly positive. The three treated nodules on the left side are very much smaller.

FIG. 5. *Macacus rhesus* X-5. The skin of the left side was X-rayed 4 days before inoculation. Picture taken 10 days after inoculation, shows nodules slightly smaller on left side than on right.

FIG. 6. Same animal, 47 days after inoculation, with two large masses on the right side (untreated), but only two small nodules on the left side (treated).




(Muller and Tyler: Effect of X-ray on verruga peruana nodules)

# IS THE LOCAL VASODILATATION AFTER DIFFERENT TISSUE INJURIES REFERABLE TO A SINGLE CAUSE?

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Plates 2 and 3

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In recent years physiologists have realized that the capillaries are capable of independent functionings. With the new knowledge have come no few hypotheses concerning the causes for capillary behavior. Important amongst these because of the fruitful experimentation and thought to which it has led is the conception that local vasodilatation in response to injury occurs through the influence of a substance derived from the damaged tissue, this substance being always the same, no matter what the kind of injury. Recently we have encountered facts which render difficult an acceptance of the hypothesis as outlined, while deserving report furthermore for themselves in this present observational stage of inquiry.

The hypothesis of a single underlying cause for all local vasodilatations was foreshadowed by the observations of Ebbecke and others, but finds its most extensive expression in the recent book of Lewis (1). Lewis has described and subjected to an enlightening analysis the "triple response" which develops after various types of skin injury. He concludes that no matter how this response is elicited it is always due to the same chemical agency (the "H substance"). "We have to deal in all instances in which an H substance is under consideration, with a single substance having an invariable chemical constitution;" and the H substance is under consideration, not merely in cases of local injury but in systemic shock, including the anaphylactic (2). Histamine elicits the triple reaction and the evidence "seems sufficient to establish the conclusion that injury of the skin liberates a substance exerting a local action indistinguishable from that of histamine upon the cutaneous nerves and vessels." So "it is difficult to refrain from stating without reserve the simple conclusion that the vasodilator substance considered and the H substance are one and the same, and that this substance is histamine, free or held in loose combination," though a reservation in the matter is maintained for the moment. The assumption that histamine is the effective cause of local pathological vasodilatation induced in many different ways, and of shock

as well, has been the stimulus to much work by others upon the amount of the substance derivable from various organs.

We shall describe experiments which prove that the local vasodilatation consequent upon mechanical injury to the skin differs from that due to histamine. In brief it has been found that the impulse to vascular contraction arising in skin from which the circulation has been cut off prevails over that responsible for local vasodilatation after mechanical injury, with result that the bloodless blanchings known as Bier's spots develop in regions where previously this vasodilatation had reigned. On the other hand the least quantity of histamine which is capable of causing local vasodilatation renders the vessels involved refractory to the contractile influence.

### *The Scratch Tests*

Volunteers were selected who yielded pronounced Bier's spots on the arm under the ordinary circumstances of venous congestion followed by arterial occlusion. The limb was propped horizontally by means of supports located beneath the elbow and wrist,—a procedure which localizes the spots to the upper side (3),—and some time prior to any development of the spots a series of scratches were made with a sterile needle after the skin had been lightly swabbed with alcohol and ether and allowed to dry. Usually two parallel scratches, from 0.6 cm. to 1.0 mm. long and 1½ to 2 mm. apart were made at intervals of 3 to 4 cm. (Figs. 1–6). Most of these were just deep enough to draw blood in some part of their length. Sometimes the skin was oiled and the progress of the vascular changes was followed with a lens or the binocular microscope.

In a first series of tests the circulation of the arm was interfered with only after the lapse of some minutes, when the characteristic responses to injury had asserted themselves. The findings were so uniform that a single protocol will suffice.

*Test 1.*—The subject, H., was a young man. The right arm, large, quite plump, with a fair, thin skin showing numerous freckles, was propped above a table, the forearm horizontal in partial supination. At five locations between elbow and wrist two scratches were made on the upper side of the arm. Those close to the elbow were in the skin that was highest, as the arm lay, the others being slantingly below this level, in proportion to their nearness to the wrist (Fig. 1). The pairs were laid down in the direction from elbow to wrist during the course of 6 minutes. Eighteen minutes later there was a well-marked, deep pink wheal, ½ cm. in transverse diameter, underlying each set of scratches, with a pink flare of active hyperemia round about and a pale zone outside (Fig. 1). Two minutes later the

pressure was raised to 70 mm. in a cuff on the upper arm, and after a further 7 minutes to 170 mm. As engorgement proceeded the vascular abnormalities in the neighborhood of the injuries became greatly accentuated (Fig. 2). The pale areola, in special, extended much further than it had previously. The skin under and about the scratches continued to be pink long after that elsewhere had turned violet. Bier's spots began to appear in the skin that was highest within 2½ minutes after the arterial occlusion; and they were especially abundant and pronounced in the regions of response to the scratching. After 12½ minutes of stasis the numerous small blanchings round about the uppermost scratches had largely coalesced and the whiteness had extended up to the scratches and even between these latter (Fig. 3). The spotting was as marked where the skin around the scratches had been red prior to the arterial occlusion as where it had been unnaturally pale (Fig. 3). To obtain Fig. 3 and the subsequent pictures the position of the camera was shifted more nearly over the lesions.

After 23½ minutes of arterial occlusion (30½ after the pressure was raised to 70 mm.) there was a complete blanching almost everywhere in the region where the two highest sets of scratches had been placed, and a less degree of it about those situated lower on the side of the arm. This blanching was complete in the sense that all blood had been driven out of the vessels. The whiteness had intruded itself between the individual scratches of the three uppermost sets and had advanced to the scratches themselves, with result that the latter stood out as dark lines on a white ground (Fig. 4).

The obstructing cuff was now released. A few of the Bier's spots unassociated with the scratches persisted for some seconds but they were soon lost, like the rest, in an active hyperemia which obliterated all previous vascular differences. Only the scratches were now to be seen on the bright red arm. During the next twelve minutes the general hyperemia gradually subsided, and the special redness under and about the scratches again became visible. It was less pronounced than before and so too with the peripheral pallor; but the whealing continued undiminished. The last photograph was taken at this time (Fig. 5).

The temperature of the room was 21°C. throughout the period of the test.

Linear scabs formed later over the scratches, remaining in place for more than a week. There were never any signs of infection.

In this test Bier's spots developed first in the regions of the scratches, and where the latter were favorably placed the contraction of the small vessels was so complete as to empty them even to the margin of the lesions themselves (Fig. 6).

The several vascular reactions occurring about scratch injuries have been carefully considered by Lewis. Our scratches were deeper than those which he employed as routine, eliciting reactions more pronounced and lasting but otherwise differing no whit from those he

has described; for the differences referable to variations in the severity of the lesion are merely those of quantity not of quality, as he remarks. The "triple reaction" (wheal, flare, local vasodilatation) was characteristically present. In the analysis of our results we shall utilize both Lewis' terminology and the many new facts that he has placed at the disposal of investigators.

On consulting Figs. 3 and 4 it will be seen that Bier's spots appeared first, and were most numerous and pronounced, in the skin regions that exhibited vascular reactions as result of the scratching. The peripheral zone of pallor became much more extensive during the period when venous blood was accumulating in the limb (Figs. 1 and 2). This may have been because the partial vascular contraction responsible for it extended further than was perceptible prior to the venous engorgement. Whatever the actual cause the early appearance of Bier's spots in this zone was to have been expected, since partial emptying of the vessels is known to favor their occurrence (4). Blanchings also developed early nearer the lesions where the skin had shown the bright hyperemia, the "flare," due to a passive widening of the small vessels consequent upon arteriolar dilatation. This is not surprising since a preliminary active hyperemia is known to enhance the tendency to spotting (5). Later tests showed that in the regions of pallor and flare about histamine lesions spotting is likewise especially pronounced, doubtless for the same reasons. Both the vascular reactions mentioned are known to be the result of indirect stimulation through nerves, not of direct action of an "H substance" upon the vessels (6).

As Figs. 4 and 6 show, the blanching in some instances extended inwards secondarily, involving the entire whealed area immediately about the scratches. At first thought it would seem remarkable that any contraction could occur of vessels so damaged that the transudation of whealing had taken place from them. But Ebbecke (7) has shown that the period of transudation lasts for but a few minutes, the extravascular fluid remaining in place for hours afterwards. Doubtless this period was over by the time the Bier's spots developed.

There remains to be considered the "local reaction" of Lewis, the vasodilatation, that is to say, which is attributed to the direct effect of the "H substance." Under the conditions described in Test 1

this reaction could not be sharply discriminated from the flare. But in the case of even the slightest of scratches the local reaction develops; and, when the lesions have the severity of those now dealt with, it extends from 1 to 2 mm. about them, as our later work showed. Its precise dimensions were unimportant in the present instance since the blanching proved completely obliterative. The local pallor could be seen to extent gradually across the linear scratches where blood did not overlie them and prevent a clear view (Fig. 6).

The instance given is typical of the phenomena encountered in individuals prone to develop well marked Bier's spots. It leaves no doubt that the contractile impulse responsible for the spots is dominant over the influence which gives rise to local vasodilatation in skin mechanically injured.

The conditions in tissue chronically inflamed do not lend themselves readily to physiological analysis, being complicated by reparatory and other changes. The red or purplish hue of the skin is often due in no small part to new-formed blood vessels.

*Test 2.*—The subject S., a middle aged man, showed on the radial side of the right forearm just above the wrist, three curving, parallel injuries from 5 cm. to 12 cm. in length, where the skin had been raked by the claws of an animal about one week previously. The lesions now had the form of raised, edematous, purply strips some 3 mm. wide, each surmounted along its axis by a firm scab approximately ½ mm. thick and high. The contrast with the pale skin round about was great. No flare or pale areola could be seen about the injuries which appeared to be uninfected and healing. The edema and the purpling of the skin nearly coincided in extent.

The subject was placed at a table with the arm propped as usual and so turned that the scratches were uppermost. The pressure in the cuff above the elbow was raised to 70 mm. for 5 minutes and then to 170. Neither during the preliminary period of congestion nor that of stasis was a flare with peripheral pallor visible about the strips. During the 30 minutes of stasis Bier's spots appeared on the upper side of the arm but not on the lower. They were abundant and pronounced, but, as the microscope showed, nowhere so complete that all blood was forced out of them. Many intruded upon the purple strips which despite the general engorgement had remained well defined, being more deeply congested than the surrounding skin; and soon these no longer appeared as strips, so interrupted by blanchings were they. In not a few places the blanched spots, as they increased in size, involved the skin on the other side of the scab as if they had extended beneath it. Yet when one compared them as a whole with the blanchings in the uninjured tissue round about one found them not so white. Evidently some slight

impediment to complete vascular contraction existed within the region that had reacted to the injury.

With the removal of the cuff all the pale spots were lost in active hyperemia, the strips standing out intensely red. In another 20 minutes this had largely subsided, and the strips were turning violet. Throughout they had remained swollen.

The foregoing test illustrates the fact that the redness of chronic inflammation will yield on occasion to the blanching of Bier's spots. We have repeatedly observed them to encroach upon the scars of small incisions that had healed long previously by granulation. But such matters lie without the province of the present work. It suffices here to bring proof (as in Test 1) that the vascular contraction which leads to Bier's spotting prevails over the local vasodilatation which develops shortly after mechanical injury to the skin.

### *The Histamine Tests*

The histamine tests were carried out as follows:—

The preparation employed was the ergamine phosphate of Burroughs Welcome and Co. When dissolved in saline and pricked into the arm according to Lewis' method it gave rise to the reactions described by this author. Sometimes it was introduced at situations where Bier's spots were known to be likely to occur in the individual utilized (Fig. 7). The highest effective dilution was in some individuals 1 in 500,000, and in others 1 in 200,000. When the arm was engorged with venous blood and the circulation cut off prior to the pricking a typical response was not always obtained even with the greater of the strengths mentioned, owing evidently, in some instances at least, to an oozing of blood from the congested skin, which must have swept out most of the histamine. To minimize this source of error several series of punctures were usually made at one time, together with control punctures of saline alone. They were placed in lines on the upper side of the horizontal forearm, which as usual was propped at elbow and wrist to facilitate the spotting. The maximum extent of the vascular reactions induced were sometimes marked out with dots of india ink.

In certain preliminary observations the histamine was punctured into the arm prior to cutting off the circulation, and the strength employed was not infrequently 1 in 1000, that is to say three times that used as routine by Lewis. It gave rise to pronounced "triple reactions." Ordinarily the Bier's spots appeared in the zones of flaring and pallor before they did in the unaffected skin, as was to

have been expected for reasons that have been sufficiently gone into; and sometimes the blanchings encroached slightly upon the wheals. Wheals, no matter how caused, tend to extend beyond the region of immediate transudation through the spread of the fluid composing them; while furthermore the vascular disturbance responsible for them is transitory, as has already been mentioned.

The essential point to be determined by the tests was whether the blanchings invaded the region of the local reaction of vasodilatation. As in the case of the scratches this merged so directly with the flare that its limits could not be defined. Therefore resort was eventually had to histamine punctures made after venous congestion and arterial occlusion had been brought about. Under such circumstances no wheal or flare develops, and the local reaction takes the form of a sharply defined purple disc with the puncture at its center. In numerous tests with graded dilutions of histamine, carried out upon several individuals, Bier's spots were never seen to intrude upon these characteristic discs though they often completely surrounded them, and proved capable of obliterating the purple dot due to control punctures of saline or to dilutions of histamine that failed to elicit the local reaction. In some of the cases studied the spotting occurred so soon after the punctures that it may well have limited the spread of the histamine within the skin (Fig. 7), the result being that the contractile impulse was opposed by that of said substance acting in a concentration which otherwise would not have obtained. However, against these instances can be placed others in which the blanchings threatened the histamine discs only after more than a half hour had elapsed, that is to say not until long after they had reached maximum dimensions. In tests upon one of the authors the circulation was sometimes cut off from the arm for 50 minutes.

It now became a crucial matter to determine the influence of Bier's spotting upon scratches laid down on the arm after venous congestion and arterial occlusion. For under such circumstances the local reaction due to the trauma might conceivably prove as obdurate as that resulting from histamine. Some evidence that this was not the case has just been mentioned, namely the obliteration by blanching of the purple dots due to puncture through saline or ineffective histamine dilutions. The following observations are conclusive.

*Test 3.*—This was made upon a middle aged man, R. In previous tests upon him histamine had proved ineffective in dilutions greater than 1 in 200,000, and this held true now when, as a preliminary, dilutions of 1 in 50,000, 1 in 100,000, 1 in 200,000 and 1 in 300,000 were pricked into the propped right forearm, about half way down on its flexor surface. Only the 1 in 50,000 gave a wheal larger than did saline alone, though the wheals resulting from 1 in 200,000 and 1 in 100,000 were somewhat more sharply defined than after saline. The 1 in 300,000 yielded a triple reaction no different from this last. After ten minutes the pressure was raised to 70 mm. Hg in a cuff on the upper arm, 5 minutes later to 170 mm., and immediately a second series of histamine punctures was laid down, this time on the uppermost side of the arm, in the skin overlying the radius. They were widely separated, and midway between each two a pair of scratches were made having the usual relations and dimensions. The row of lesions now extended from near elbow to near wrist, the interval between them averaging about 2½ cm. All this took 3¾ minutes. A little more than a minute later the first slight mottling with Bier's spots had begun to appear here and there. Only the 1 in 50,000 and 1 in 100,000 histamine dilutions gave rise to the characteristic purple discs of vasodilatation and only that caused by the latter dilution became surrounded by Bier's spots during the 48 minutes of stasis. These spots had coalesced about it within 15 minutes, forming a uniform white background against which it stood out sharply; but the blanching never encroached upon it in the least. The local purpling around and between the parallel scratches, on the other hand, was replaced by blanching in several instances.

At once more extensive tests were undertaken on the left forearm, which was propped thumb up. The veins from the limb were first obstructed for 7 minutes at 70 mm. Hg pressure and then the arteries as well, at 170 mm. As rapidly as possible thereafter 5 series of histamine punctures were made on the radial side of the arm, with the 1 in 50,000, 1 in 100,000, 1 in 200,000 dilutions only, since these alone had previously proven effective. One series was laid down at a time in order of progressive histamine strengths and the needle was well cleansed before the next series was proceeded to. An assistant charted the situation of the punctures. The entire procedure took 3 minutes. Two series of the punctures were placed at about 4 cm. intervals in a line on the skin that was uppermost (that over the radius). The other series were located high on the flexor surface of the forearm, the punctures being somewhat closer together. Next, scratches were rapidly made in pairs between each two of the widely separated punctures over the radius and also beyond them at the wrist. This required another minute. There were seven pairs of scratches in all.

Bier's spots began to appear after the arterial occlusion had endured 7 minutes, but not in situations that threatened the histamine discs and the scratch reactions until after more than 15 minutes. By that time the spotting had crossed a tiny isthmus of undamaged skin existing midway in one of the scratches. Two small and faint discs had resulted from the 5 punctures with 1 in 200,000 histamine, 3

from the 5 with the 1 in 100,000 dilution, and 5 from 5 with the 1 in 50,000. These last were pronouncedly purple. No more developed during the succeeding 41 minutes before the circulation was restored to the arm after 56 minutes of stasis in all. During this time Bier's spots became numerous, large and brilliant. They merged and completely surrounded one of the two characteristic discs produced by the 1 in 200,000 histamine dilution but never encroached upon it; and they extended to the periphery of the other disc on one side without ever affecting its shape. The discs caused by the greater strengths of histamine likewise proved resistant to blanching. The local reaction next the scratches, on the other hand, which was manifest as an intense purpling between them and for approximately 2 mm. around them, had yielded to the blanching at the numerous places where this impinged upon it; and here and there the latter reached the scratches themselves and extended between them.

Special care was taken in this and other similar tests to determine whether the local reactions of histamine and mechanical injury tended to persist when the circulation once again entered the arm. On the present occasion both disappeared promptly in the reactive hyperemia.* When the latter had largely subsided, after 21 minutes, a wide reddening was visible about the scratches and a whealing immediately next them. No histamine reactions whatever could now be discerned. Next day the scratches bore linear scabs and the tissue for a few mm. about them was hyperemic and edematous. Healing was prompt.

During the period of circulatory obstruction the engorged vessels in the areas of local reaction about the scratches had the same appearance under the binocular microscope as those in the histamine discs.

Important for the interpretation of such tests as this is the question whether the dilated vessels of the purple discs induced by histamine may not have proved obdurate to blanching because of an impacted content of red cells. For it is known that under ordinary conditions histamine causes small vessels to become clogged with cells, the plasma passing out into the tissues. But in instances such as the foregoing, in which the circulation was interrupted and whealing absent, there was no opportunity for corpuscles to accumulate in the dilated vessels by escape of plasma from them. They flushed promptly when blood was let into the arm.

* It has been our experience that histamine diluted with Ringer's solution instead of saline is prone to produce discs which persist during reactive hyperemia and do not fade even when pressed upon with a glass slide when the hyperemia is at flood. Clotting must take place in the distended vessels. An intravascular clotting can also be produced by trauma, of course. Mere puncture with a needle sometimes gives rise to a purpling resistant to pressure and occasionally this was observed here and there along scratches such as we have employed.

The data obtained justify the conclusion that the local vasodilatation induced by minimal amounts of histamine resists the blanching of Bier's spots, whereas that caused by relatively severe mechanical injury in the same subject yields readily before it.

## DISCUSSION

When pituitary extract or epinephrin is injected into regions of local vasodilatation due to acute inflammation no contraction of the vessels occurs (8); and yet, as we have shown, they yield before the contractile influence that is responsible for Bier's spotting. The fact is the more remarkable because this influence would seem to be but weak, proving ineffective ordinarily on the under side of the engorged arm, because the weight of the blood gravitating to the region acts to oppose it there (9).

The local vasodilatation produced by histamine is likewise refractory to pituitary extract or epinephrine (10) and this has been taken to corroborate the view that it is caused by the same "H substance" as the reaction at sites of inflammation (11). That the inference is unwarranted would seem to be sufficiently proven by the failure of the histamine reaction to yield before Bier's spotting. It may be objected that the tests with histamine involved the introduction of this substance from without, whereas the H substance of inflamed tissue is formed *in situ*. But the hypothesis of an H substance, in so far as it concerns histamine, rests on the basis of tests with this substance introduced from without. Furthermore in our experimental comparison of the resistance of the local reactions about scratches and histamine punctures, respectively, to Bier's spotting, all the conditions have been such as would in themselves tend to render the histamine reaction greatly the more susceptible. For the minimum effective quantity of histamine that was introduced by puncture, though persisting with undiminished effectiveness during short periods of stasis (12), must surely have been dissipated in part, by diffusion if in no other way, during the long periods of the present experiments. When the circulation was restored to the arm no whealing occurred at the site of puncture, nor any flare or local reaction after the reactive hyperemia had subsided. It is possible that this hyperemia may have flushed the histamine away or have lasted so long as to have masked

completely the transitory vasodilatation caused by the substance. With the scratch injury, however, no such difficulties of interpretation are met. Granting that the H substance causes the local reaction about the scratch, one must suppose either that this substance is formed in very large quantity to begin with or else continually in small amount; for otherwise the local reaction could not persist throughout many hours in an arm with good circulation, and survive prolonged hyperemia after stasis. On either supposition the local reaction due to the scratching should have proved the more obdurate to Bier's blanching. But always it yielded promptly whereas the histamine dilatation remained absolutely refractory.

Brief mention has been made of tests which showed that Bier's spots can invade old scars which have healed by granulation. Tests upon the vessels in new-formed granulation tissue may throw much light on the mechanism whereby the spots come about. The cutaneous pallor supervening upon death is due to a vascular contraction of the sort responsible for them. This pallor is said by morbid anatomists to involve granulation tissue as well as the normal skin; but the point deserves close study.

SUMMARY

Experiment shows that the vascular contraction responsible for Bier's spotting prevails over the local vasodilatation due to mechanical injury of the skin, and causes the local reddening to blanch. It is without effect, however, upon the local vasodilatation caused by histamine pricked into the skin. The results raise doubts concerning the validity of the hypothesis referring all local vasodilatations to the action of a single chemical substance or set of substances ("H substance"), liberated within the tissues.

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## EXPLANATION OF PLATES

The pictures were all taken by Cooper Hewitt light. While giving strong contrast this emphasizes the red ingredient only of any violet hue that may be present.

### PLATE 2

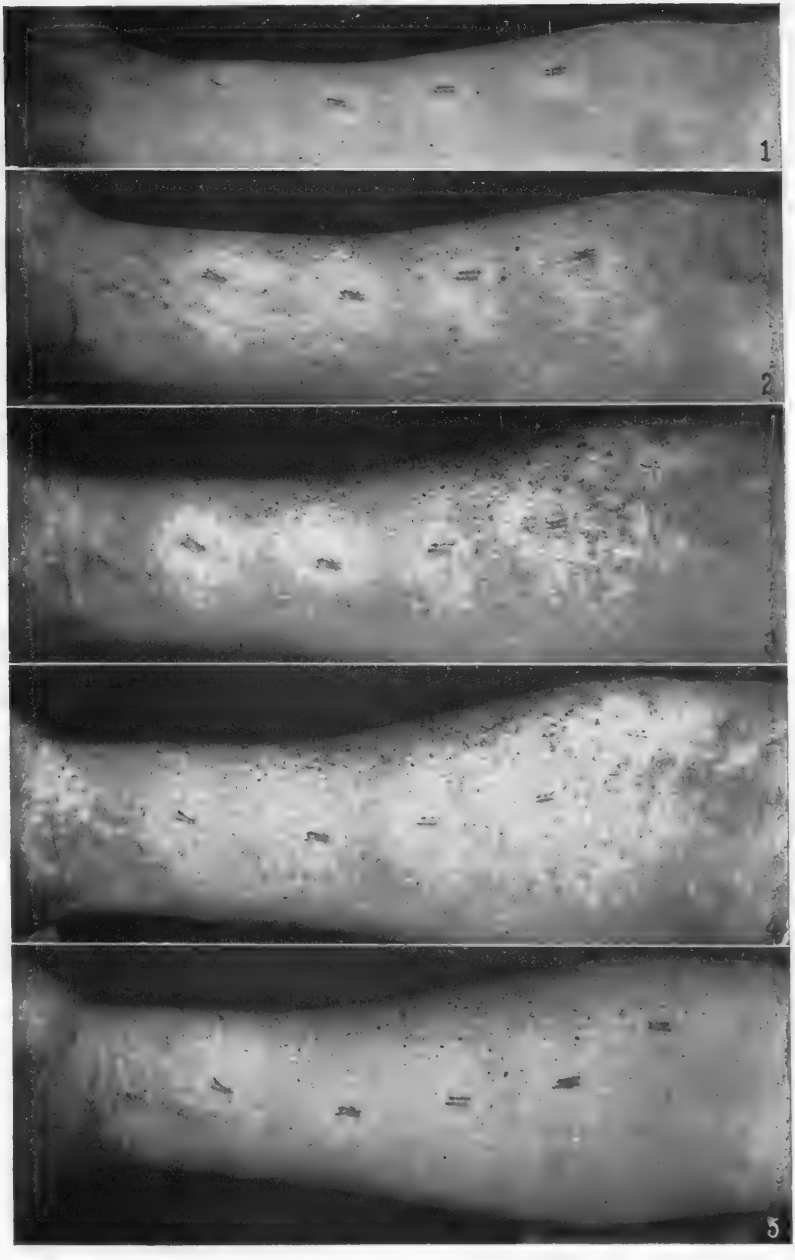
FIGS. 1–5. To show that the vasoconstriction of Bier's spotting prevails over the local vasodilatation caused by mechanical injury.

Parallel scratches just sufficiently deep to draw blood were made at five locations upon the right forearm. Fig. 1 shows the condition of affairs 20 minutes later. There is a spreading hyperemia with peripheral pallor and a well-marked wheal immediately about the scratches. Fig. 2 was obtained 5 minutes later, after the veins from the arm had been shut off for 3 minutes by 70 mm. pressure in a cuff on the upper arm. The hyperemia appears practically unchanged but the pallor round about it is more widespread. Pressure was maintained on the veins for another 4 minutes and then raised to 170 mm., cutting off the circulation entirely. Within the succeeding 12½ minutes Bier's spots became abundant in the regions of vascular reaction about the scratches, and extended even into the narrow space between scratches that lay parallel, as Fig 3 shows. For the purpose of this figure and later ones the camera was shifted so as to be almost directly above the partially supinated forearm. The photograph of Fig. 4 was taken after the circulation had been cut off for 23½ minutes. Pressure in the cuff on the upper arm was now relaxed. 12 minutes later, when the active hyperemia had largely subsided, Fig. 5 was obtained. It shows the whealing and local reaction that were noted prior to experiment, but the flaring hyperemia and pallor were less evident than before.

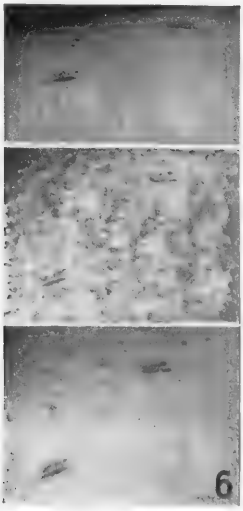
### PLATE 3

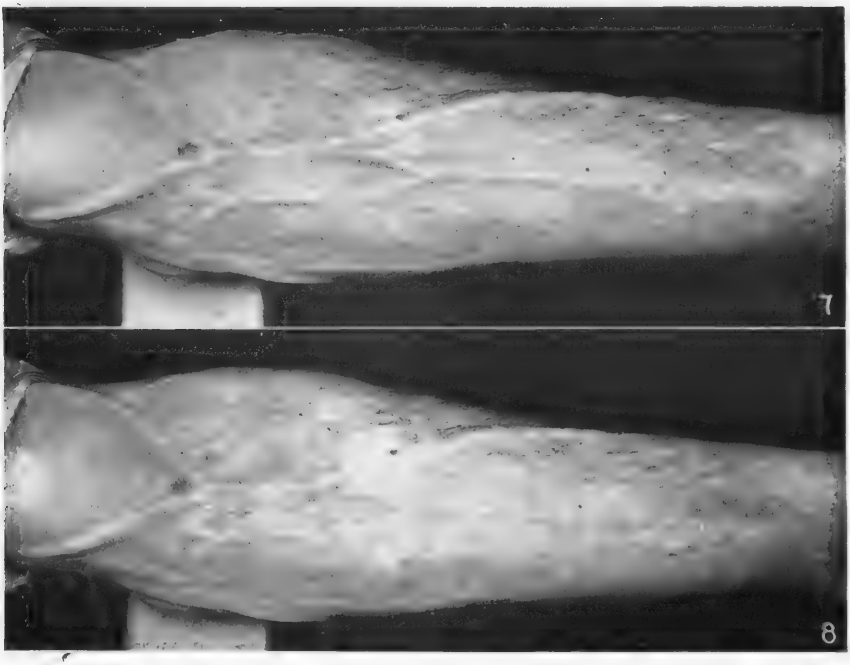
FIG. 6. Enlargements of portions of Figs. 1, 4 and 5, to show the complete replacement by blanching of the vasodilatation next some of the scratches. Successive phases are here depicted of the reaction occurring about the two pairs of lesions that were highest on the arm, and hence most encroached upon by Bier's spots.

FIGS. 7 and 8. To show the obduracy of the vasodilatation induced by histamine. It had previously been found that the subject of this test was prone to develop a large Bier's spot over the radial basilic vein of the supinated forearm and a smaller one over the median basilic. Histamine in 1 in 1000 solution was pricked into the skin over the latter vein and seven minutes later a general venous obstruction was produced with a cuff on the upper arm. By this time an enormous wheal, with reddening and a flare, had developed about the needle puncture. 4½ minutes later the circulation to the arm was completely cut off and after another minute the histamine dilution was pricked into the skin over the radial basilic vein. Bier's spotting developed here almost as rapidly as did the purple histamine disc and soon it surrounded and sharply demarcated the latter. Fig. 7 was obtained after the circulation had been cut off 11 minutes, and Fig. 8 after 18 minutes more. The shape of the histamine disc had remained absolutely the same in the interval despite the progressive spread and intensification of the blanching about it. The test had only the use of furnishing a picture to illustrate the characteristic demarcation of histamine discs by Bier's spots. For not only was the amount of histamine pricked in several hundred times the minimum effective quantity but the rapid occurrence of vasoconstriction (Bier's spotting) about the disc while it was still developing must have served to retain the substance in concentrated form close to its original locus.




(Rous and Gilding: Cause of local vasodilatation)




(Rous and Gilding: Cause of local vasodilatation)

# AN APPARATUS FOR THE STUDY OF RESPIRATORY QUOTIENT AND BASAL METABOLISM OF MICE

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During the course of some investigations in this laboratory, it became necessary to ascertain the respiratory quotient and basal metabolism of mice. Therefore, a study was made of an apparatus and procedure which could be readily applied to a large number of animals, and would yield precise results.

Apparatus for determining respiratory quotients fall into two classes, the open-circuit type and the closed-circuit type. In the former, a stream of dry air freed from carbon dioxide is passed through a chamber containing the animal. The water vapor and carbon dioxide in the outgoing air are absorbed and weighed. At the same time, the loss in weight of the animal and the weight of its container are noted. The amount of oxygen used is then determined indirectly as the difference between the combined weights of water and carbon dioxide given off and the loss in the weight of the animal. Considerable precaution must be taken in weighing the aborption vessels and animal chamber. Haldane[1] was among the first to apply this procedure to the study of respiratory exchange of small animals, so that the method is commonly known by his name. This apparatus was also used by Pembry[2] in studying the rate of respiration of mice at various temperatures, and by Moog[3] for determining the respiratory quotient of guinea pigs. It has been somewhat modified and greatly complicated by Murschauser,[4] who removed the carbon dioxide by means of an elaborate system of absorption bottles containing barium hydroxide solution, and determined the oxygen consumed by analysis of samples of the expired air. More recently, Aszodi[5] has used a simple apparatus of this type for the study of induced hibernation in white mice. Several experiments in which the oxygen consumed was ascertained directly as well as indirectly showed that the values obtained by the two methods agreed to within 2

[1] Haldane, J. S., *J. Physiol.*, 1891, **13**, 419.

[2] Pembry, M. S., *J. Physiol.*, 1893, **15**, 401.

[3] Moog, R., *Compt. rend. Soc. biol.*, 1911, **71**, 520.

[4] Murschauser, H., *Biochem. Z.*, 1912, **42**, 262.

[5] Aszodi, Z., *Biochem. Z.*, 1921, **113**, 70.

per cent. Aszodi's apparatus has been applied to the metabolism of white rats by Goto,[6] Asada,[7] and Händel and Tadenuma.[8] A very simple continuous-flow apparatus has been described by Artundo,[9] for use with rats and rabbits in which the respiratory exchange is determined by analysis of samples of the expired air by means of a Haldane gas analysis apparatus.

In the closed-circuit type of apparatus, the respired air is passed through an absorbent to remove the carbon dioxide and then returned to the respiration chamber to be used over again. Oxygen is supplied as needed to replace that taken up by the animal. Thus, the oxygen consumed is measured directly by the quantity required to maintain constant pressure in the system. The amount of carbon dioxide given off is obtained by weighing the absorption vessels or by titration. An apparatus of this type was first applied to the study of small animals by Regnault and Reiset.[10] Zuntz[11] has described an exceedingly complicated apparatus operating on this principle which was later modified by Zuntz and Oppenheimer.[12] Elsas[13] studied the respiratory exchange of frogs, using an apparatus in which air was circulated by a motor-driven pump, and the carbon dioxide absorbed in barium hydroxide solution was determined by titration. The oxygen was measured as introduced from a gasometer and checked by analysis of samples of the respired air. The experimental period extended over 20 to 24 hours. He considered the average error to be about 2 per cent. The same apparatus was used by Lesser[14] for the determination of the respiratory quotients of mice. He reported that the error in the quotients obtained did not exceed 0.02. Hildebrandt[15] used a similar apparatus for the study of the respiration of white rats. A model described by Benedict[16] for use with rabbits and guinea pigs includes special provision for recording movement of the animal and for introducing food. It is designed for use over a period of 24 hours or longer. A very elaborate apparatus for experiments lasting a week or more has been constructed by Kolls and Lovenhart.[17] Rubner[18] has described a comparatively simple device for use with animals weighing from 20 to 60 grams. Air is circulated by means of a rotary

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[6] Goto, K., *Biochem. Z.*, 1923, **135,** 107.
[7] Asada, K., *Biochem. Z.*, 1923, **143,** 387.
[8] Händel, M., and Tadenuma, K., *Z. f. Krebsforschung.*, 1924, **21,** 197.
[9] Artundo, A., *Compt. rend. Soc. biol.*, 1927, **97,** 407.
[10] Regnault, V., and Reiset, J., *Ann. Chim. Phys.*, 1849, **26,** 299.
[11] Zuntz, N., *Arch. f. Physiol., Suppl.*, 1905, p. 431.
[12] Zuntz, N., and Oppenheimer, C., *Biochem. Z.*, 1908, **14,** 361.
[13] Elsas, B., *Z. f. Biol.*, 1913, **62,** 3.
[14] Lesser, E. J., *Biochem. Z.*, 1924, **153,** 39.
[15] Hildebrandt, F., *Arch. exp. Path. u. Pharm.*, 1922, **92,** 68.
[16] Benedict, F. G., *J. Biol. Chem.*, 1915, **20,** 301.
[17] Kolls, A. C., and Lovenhart, A. S., *Am. J. Physiol.*, 1915, **39,** 67.
[18] Rubner, M., *Sitzb. preuss. Akad. Wiss.*, 1924, p. 2.

pump. Oxygen is supplied by electrolysis, the volume added being determined by measuring the volume of hydrogen evolved at the cathode. The duration of an experiment is about 24 hours. Other apparatus for use with small animals have been described by Foster and Sundstroem,[19] Wesson,[20] and Schoeller, Gehrke, and Michael.[21] Fridericia[22] has described a closed-circuit apparatus in which the oxygen consumed can be determined by two methods, thus obtaining a double check on this value. Cori and Cori[23] have used the apparatus of Fridericia in a study of the metabolism of rats. Agreement between the values of direct and indirect oxygen determinations averaged 1.4 per cent. They consider the value for the respiratory quotient to be accurate to ± .008.

Although many of the apparatus cited above are applicable to the study of the metabolism of mice, none are particularly adapted to rapid routine manipulation. The difficulties encountered in keeping a large circulatory system perfectly air tight have been emphasized by many workers. Any procedure involving methods of exact gas analysis requires particular care and is very time consuming, as is true of methods involving accurate weighing of gas absorption vessels. An attempt to develop a procedure which will avoid these disadvantages, and at the same time provide a method for the measurement of the respiratory quotient with sufficient accuracy, has resulted in the apparatus and technique described in this paper.

The apparatus (Fig. 1) consists of a closed glass chamber (A) of about 500 cc. capacity, in which a wire cage (B) is suspended. The carbon dioxide given off by the animal is absorbed by an N/20 solution of barium hydroxide. This solution is introduced into the chamber from burette (C) at the beginning of the experiment. At the conclusion of the experiment, the excess barium hydroxide is titrated with an N/50 solution of oxalic acid contained in burette (D). An air-propelled fan and glass paddle mounted on the same shaft circulate the air and thoroughly stir the liquid. The shaft of the stirrer enters the chamber through a mercury seal (E). The chamber is ventilated before and after the experiment by aspirating a current of air through tubes (F) and (G). Variations in the pressure during the run are

[19] Foster, G. L., and Sundstroem, E. S., *J. Biol. Chem.*, 1926, **69**, 565.
[20] Wesson, L. G., *J. Biol. Chem.*, 1927, **73**, 499.
[21] Schoeller, W., Gehrke, N., and Michael, S., *Biochem. Z.*, 1927, **189**, 220.
[22] Fridericia, L. S., *Biochem. Z.*, 1913, **54**, 92.
[23] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1926, **70**, 557.

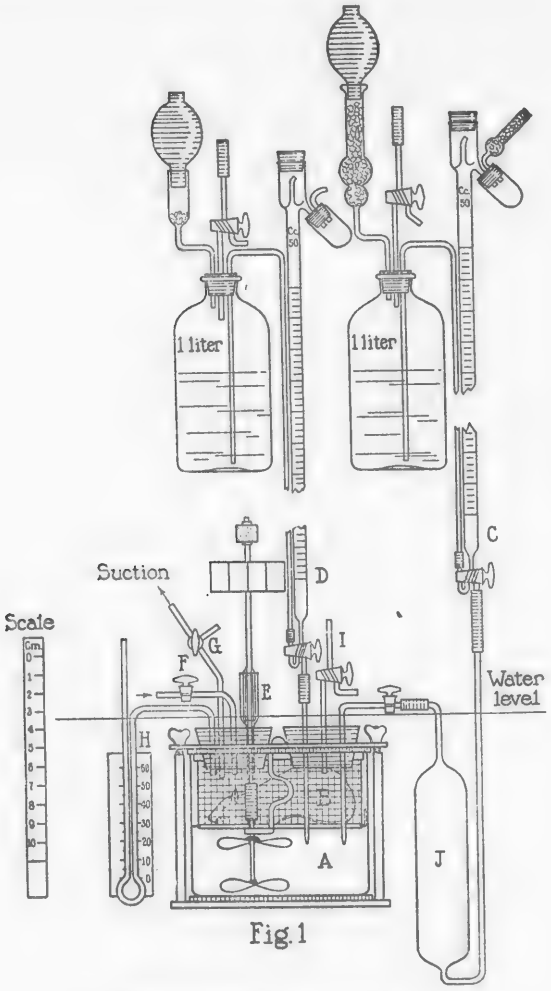


Fig. 1

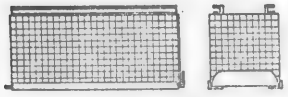

Fig. 2

recorded on the manometer (H). When the chamber is closed, the animal consumes oxygen and the pressure in the chamber is reduced. Compensation is made by running in an equivalent volume of barium hydroxide solution so that the amount of oxygen consumed is measured by the volume of barium hydroxide solution required to maintain atmospheric pressure within the chamber. The volume of carbon dioxide given off during the same period is calculated from the titration. The respiratory quotient is then obtained by dividing this volume of carbon dioxide by the volume of oxygen consumed. Proper functioning of the apparatus can be checked at any time by running a blank experiment in which a measured amount of pure, dry carbon dioxide is passed into the chamber. Capillary stop-cock (I) is provided for this purpose. The brass plate which carries the cage, together with all inlets and outlets to the chamber, is fixed rigidly to an iron stand which also supports the burettes and reservoirs. The removable wire cage (Fig. 2) is provided with a sliding floor for the introduction of the animal. The glass chamber rests on a brass plate which is clamped to the fixed plate by thumb screws. The fixed plate carries a rubber gasket which insures an air-tight seal. The chamber and reservoir (J) are immersed in a water thermostat. The purpose of this reservoir is to eliminate temperature changes in the barium hydroxide solution when it is introduced into the chamber. Absolute quiescence of the animal throughout the experiment is assured by an intraperitoneal injection of "luminal sodium."

### *Experimental Procedure*

The animal is injected intraperitoneally with 0.10 cc. of a 2 per cent solution of "luminal sodium," and immediately placed in the wire cage which is then suspended under the fixed plate. Since, during the experiment, the air of the chamber is in contact with a dilute aqueous solution, provision must be made for saturating it with water vapor during the preliminary period. Otherwise the vapor pressure, which would develop as soon as the chamber should be closed, would introduce error into the oxygen determination. To this end, 5 cc. of water are placed in the chamber together with a few drops of phenolphthalein indicator necessary for the subsequent titration. The chamber is then clamped firmly into place. The fan is started, and the suction adjusted so as to provide a current of air at the rate of about 1 liter per minute. For the reasons just mentioned, the air used for ventilation must not only be at the same temperature as the thermostat but must also be saturated with water vapor. The incoming air, therefore, circulates

through a copper coil and humidifying device immersed in the bath. The temperature and humidity of the incoming air may easily be checked by closing the chamber and observing any changes in the manometer. About 30 minutes are required to reach thermal equilibrium. However, the actual run is not started until one hour has elapsed from the time of the injection, so that the animal will be thoroughly under the influence of the drug. With the current of air still passing through the chamber, about 30 cc. of the barium hydroxide solution are introduced, stop-cock (G) is turned to divert the suction to the open air, and stop-cock (F) is closed, thus sealing the chamber. The time of closure is recorded and the barium hydroxide burette is read. As the oxygen is consumed by the animal, the pressure in the chamber decreases, as indicated by the manometer. Barium hydroxide solution is run in as required to restore atmospheric pressure. At the end of about 30 minutes, the time is again recorded, stop-cock (F) is opened, suction is restored, and the excess barium hydroxide titrated rapidly with oxalic acid. The volume of oxygen consumed is given directly by the volume of barium hydroxide solution introduced during the run. From the titration data, the temperature of the bath, and the barometric pressure, the volume of carbon dioxide given off by the animal is computed. Data and calculations from a typical experiment follow.

| | | | |
|---|---|---|---|
| Weight of animal | 23.3 gm. | Normality of $Ba(OH)_2$ | 0.0491 |
| Temperature of bath | 28°C. | Normality of $H_2C_2O_4$ | 0.0204 |
| Barometric pressure | 760 mm. | Duration of experiment | 28 minutes |

| | |
|---|---|
| $Ba(OH)_2$ introduced at start | 30.00 cc. |
| $Ba(OH)_2$ added to compensate for oxygen consumed | 21.55 cc. |
| Total $Ba(OH)_2$ added | 51.55 cc. |
| Volume of $H_2C_2O_4$ equivalent to 51.55 cc. of $Ba(OH)_2$ | 123.72 cc. |
| " " $H_2C_2O_4$ required to titrate excess $Ba(OH)_2$ | 59.35 cc. |
| " " $H_2C_2O_4$ equivalent to $CO_2$ absorbed | 64.37 cc. |
| Volume of $CO_2$ absorbed at standard conditions as calculated from above | 14.64 cc. |
| Volume of $CO_2$ absorbed at 760 mm. and 28°C. | 16.14 cc. |
| Volume of $O_2$ consumed at 760 mm. and 28°C. (vol. $Ba(OH)_2$ added) | 21.55 cc. |

$$\text{Respiratory quotient} = \frac{16.14}{21.55} = 0.749$$

### *Standardization of the Apparatus*

The accuracy of the apparatus is dependent upon the extent to which the carbon dioxide absorbed represents the carbon dioxide given off by the animal during the time of the run. The degree to which this condition is fulfilled may be tested by introducing a known

volume of pure carbon dioxide under circumstances which simulate as nearly as possible the conditions of the experiment.

TABLE I

*Standardization of Apparatus by Means of Pure Carbon Dioxide*

| Experiment No. | Rate of introduction of $CO_2$ | Volume of $CO_2$ | |
|---|---|---|---|
| | | Introduced | Titrated |
| | *cc. per minute* | *cc.* | *cc.* |
| 1 | 0.47 | 15.00 | 14.91 |
| 2 | 0.48 | 15.00 | 15.06 |
| 3 | 0.51 | 15.30 | 15.24 |
| 4 | 0.8 | 20.00 | 20.20 |
| 5 | 0.8 | 20.00 | 20.20 |
| 6 | 1.1 | 16.20 | 16.70 |
| 7 | 1.8 | 21.00 | 21.50 |
| 8 | 1.9 | 20.00 | 20.20 |
| 9 | 3.2 | 20.00 | 19.25 |

TABLE II

*Respiratory Quotients of Fasted Mice*

| Exp. No. | Animal | Weight | Duration of run | $O_2$ consumed | $CO_2$ evolved | R. Q. $CO_2/O_2$ |
|---|---|---|---|---|---|---|
| | | *grams* | *minutes* | *cc.* | *cc.* | |
| 1 | 1 | 19.30 | 37 | 20.00 | 14.10 | 0.705 |
| 2 | 1 | 19.30 | 33 | 20.00 | 14.35 | 0.716 |
| 3 | 2 | 25.50 | 33 | 20.20 | 13.90 | 0.688 |
| 4 | 2 | 25.50 | 37 | 20.20 | 14.27 | 0.706 |
| 5 | 3 | 28.60 | 31 | 20.30 | 14.10 | 0.694 |
| 6 | 3 | 28.60 | 33 | 20.55 | 14.85 | 0.723 |
| 7 | 4 | 26.40 | 27 | 20.60 | 14.32 | 0.696 |
| 8 | 4 | 26.40 | 31 | 20.05 | 14.50 | 0.725 |
| 9 | 5 | 20.50 | 34 | 20.00 | 14.17 | 0.708 |
| 10 | 5 | 20.50 | 35 | 20.25 | 14.04 | 0.695 |
| 11 | 6 | 22.00 | 36 | 20.05 | 14.62 | 0.729 |
| 12 | 6 | 22.00 | 35 | 20.00 | 14.62 | 0.731 |
| Average | | | | | | 0.710 |

The apparatus is arranged in the thermostat and a stream of carbon dioxide from a gas burette is passed into the chamber through the capillary stock-cock (I). The rate of introduction of the gas is adjusted to duplicate as nearly as possible

the rate at which carbon dioxide is normally expired by a mouse, as determined in previous experiments. In order to be certain that variations in the rate of production of carbon dioxide, which might be expected in normal animals, were without appreciable effect upon the validity of the data, carbon dioxide was introduced at rates varying from 0.5 to 3 cc. per minute. The stream of carbon dioxide is allowed to pass into the chamber during a preliminary period of about 20 minutes. 50 cc. of barium hydroxide solution are run into the chamber, this quantity being approximately the total amount used in a determination upon an animal. The chamber is then closed. The stream of carbon dioxide is continued until about 15 to 20 cc. have been introduced. Ventilation is reestablished and the excess barium hydroxide titrated. A comparison of the carbon dioxide absorbed with the carbon dioxide introduced, as read on the gas burette, serves as an indication of the accuracy of the apparatus. Data obtained in this manner are given in Table I.

The operation of the apparatus was further checked by determining the respiratory quotients of mice which had been fasting for 36 hours. It has been shown that fasted rats give a respiratory quotient of 0.71 to 0.72, and Benedict[24] has recommended their use for the standardization of apparatus employed in the study of metabolism of small animals. Duplicate runs were made in each case. Data obtained are given in Table II.

## DISCUSSION

The apparatus and procedure here outlined are, in some respects, distinct departures from the methods heretofore employed. In the first place, the animal is confined in a chamber where the atmosphere is saturated with water vapor. Although it has been shown[25] that above 25°C. the effect of increased humidity is to raise the metabolic rate, there is little reason for believing that it would have any pronounced effect on the respiratory quotient. Values for the respiratory quotient of normal animals determined in this apparatus appear to confirm this inference.

In other apparatus of this type, the composition of the air is kept practically constant throughout the experiment by introducing pure oxygen to replace that consumed. This necessarily involves complications in the apparatus which can be avoided if the oxygen content be allowed to decrease within certain limits. It has already been

[24] Benedict, F. G., in Abderhalden, E., Handbuch der biologischen Arbeitsmethoden, Berlin, 1926, Abt. IV, Teil 10, 537.

[25] Cluzet, J., *Compt. rend. Acad.*, 1908, **146**, 773.

shown[26, 27, 28, 29] that a decrease in the oxygen content of respired air is without noticeable effect upon respiration as long as it does not fall below 10 to 12 per cent. In the present procedure, the concentration of oxygen in the chamber is never less than 15 per cent, so that the metabolism of the animal should at no time be affected by oxygen want.

The success of the carbon dioxide determination depends upon the extent to which the amount of carbon dioxide, computed from the titration, represents the amount of carbon dioxide given off by the animal during the time of the experiment. If air is passed through the chamber at the rate of one liter per minute, the concentration of carbon dioxide, initially present, will be from 0.5 to 1 cc. per liter. Experiments in which pure carbon dioxide was introduced into the chamber indicated that with this initial concentration the carbon dioxide was taken up by the barium hydroxide as fast as it was introduced, so that, at the close of the run, the concentration of carbon dioxide was practically unchanged. Therefore, the carbon dioxide present at the beginning is compensated for by the same amount of carbon dioxide left unabsorbed at the end of the run. This being the case, the carbon dioxide absorbed will be an accurate measure of the carbon dioxide given off by the animal during the time of an experiment.

The volume of the chamber necessarily imposes certain limits upon the total amount of fluid introduced. Although the accuracy of a titration is favored by the use of dilute solutions, it is essential that the barium hydroxide solution be of a sufficient concentration to produce rapid absorption of carbon dioxide. It was found that an N/20 solution best fulfilled these requirements. The limited space available for titration did not make it feasible to use an acid solution of a greater dilution than N/50.

The accuracy of the carbon dioxide determination will be affected by the speed at which the barium hydroxide solution is introduced at the beginning of the run, and the time required to titrate the excess

[26] Friedländer, C., and Herter, E., *Z. A. physiol. Chem.*, 1879, **3**, 19.
[27] Fraenkel, A., and Geppert, J., *Compt. rend. Acad.*, 1883, **96**, 1740.
[28] Terray, P., *Pflüger's Arch.*, 1896, **65**, 393.
[29] Durig, A., *Arch. f. Physiol., Suppl.*, 1903, p. 209.

barium hydroxide at the end of the run. However, by the exercise of reasonable precaution, error from this source should be negligible.

From a study of the data in Table II, it appears that the probable error of a single observation is 0.015, and the probable error of the arithmetical mean is 0.004. These data, together with the results obtained in the standardization of this apparatus with pure carbon dioxide, indicate that the values for the respiratory quotients obtained are accurate within 0.02. The mean value obtained from a series of such observations should be readily brought to an accuracy of 0.01.

## CONCLUSIONS

A simple apparatus has been developed for the study of the respiratory quotient and basal metabolism of mice. Data are given which indicate that the values for the respiratory quotient obtained are accurate to within 0.02. The apparatus is especially designed for rapid, routine manipulation.

# THE EFFECT OF UNILATERAL NEPHRECTOMY ON THE SENILE ATROPHY OF THE KIDNEY IN THE WHITE RAT

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(Received for publication, October 26, 1929)

Arataki (1) has demonstrated that there is a decrease in the number of glomeruli in the kidney of the white rat in old age. According to Arataki (1) this senile loss of glomeruli begins after 350 days of age and reaches two-thirds of the adult value at 500 days of age. Since this atrophy is not accompanied by gross or histologic evidence of pathologic destruction of the glomeruli, we may tentatively consider it as physiologic.

Arataki (2), Jackson and Shiels (3) Moore (4) and others have demonstrated that unilateral nephrectomy during the growth period (1 to 150 days of age) has no effect on the total number of glomeruli in the adult kidney. We have found no published reports concerning the effect of unilateral nephrectomy on senile atrophy.

## *Method*

Thirty-two white rats from the colony of the Dept. of Zoology of Yale University were used, and are designated as the Yale Strain.† All animals were 200 days of age at the beginning of the experiment. The right kidney was removed from 16 animals by the usual lumbar route, under ether anesthesia. The remaining 16 animals were used as controls. Two animals from each group were sacrificed by a blow on the head at intervals of 30 days and the left kidney was injected with Janus Green B according to the technique of Nelson (5). The right kidney of the control animals was fixed in Zenker formol, embedded in paraffin and sectioned for histologic examination and measurement of glomeruli.

The total number of glomeruli in the injected kidneys was counted according to the technique of Nelson (5). In all cases the counts are complete enumerations of the left kidney.

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* Hanna Research Fellow in Pathology.

† This designation is necessary, since in the quantitative studies on the kidney carried on in this laboratory, three strains of white rats have been used.

TABLE I

*Nephrectomy at 200 Days of Age*

| Experimental | | | Control | | |
|---|---|---|---|---|---|
| Rat No. | Count | Weight | Rat No. | Count | Weight |
| Sacrificed 230 days (Dec. 1–1928) | | | | | |
| | | *gms.* | | | *gms.* |
| 3 | 28,756 | 195 | 23 | 28,092 | 170 |
| 4 | 41,427* | 215 | 18 | 40,369* | 216 |
| Sacrificed 260 days (Jan. 1–1929) | | | | | |
| 1 | 28,142 | 195 | 20 | 24,299 | 193 |
| 2 | 26,000 | 182 | 17 | 25,745 | 185 |
| Sacrificed 290 days (Feb. 1–1929) | | | | | |
| 5 | 20,569 | 245 | 22 | 21,693 | 253 |
| 6 | 25,121 | 223 | 25 | 26,491 | 223 |
| Sacrificed 320 days (Mar. 1–1929) | | | | | |
| 7 | 18,549 | 248 | 24 | 17,272 | 225 |
| 8 | 22,687 | 200 | 29 | 21,541 | 200 |
| Sacrificed 350 days (April 1–1929) | | | | | |
| 9 | 21,870 | 210 | 27 | 24,093 | 210 |
| 10 | 22,333 | 205 | 18 | 24,538 | 205 |
| Sacrificed 380 days (May 1–1929) | | | | | |
| 11 | 22,723 | 185 | 26 | 22,591 | 185 |
| 12 | 21,504 | 185 | 19 | 22,526 | 200 |
| Sacrificed 410 days (June 1–1929) | | | | | |
| 13 | 17,727 | 153 | 30 | 23,727 | 157 |
| 14 | 28,967* | 195 | 28 | 24,227 | 200 |
| Sacrificed 440 days (July 1–1929) | | | | | |
| 15 | 29,889* | 158 | 32 | 22,939 | 158 |
| 16 | 21,725 | 180 | 31 | 22,116 | 177 |

* These animals are of a different strain. See discussion in the text.

## RESULTS

The results may be divided into two series of observations, first, the total glomerular counts, and second, glomerular sizes.

Inspection of Table I and Fig. I reveals that control animals with a count of about 28,000 at 230 days of age gradually decline to a count of 18,000 to 22,000 at 320 days of age. After 320 days of age there is little further loss of glomeruli. This is in general agreement with the results of Arataki (1).

It is further quite evident that there is no significant variation between the control and experimental animals. The experimental method precludes any quantitative observations on increase in

TABLE II

| Age | Size of glomeruli (microns) | | |
|---|---|---|---|
| | Minimum | Maximum | Average |
| *days* | | | |
| 230 | 70 | 123 | 95.2 |
| 440 | 38 | 157 | 103.6 |

TABLE III

| Age | Average diameter of glomeruli (microns) | Total number of glomeruli | Total glomerular surface mm.² ($4\pi r^2$ = surface) |
|---|---|---|---|
| Adult | 95.2 | 28,092 | 799.7 |
| Senile | 103.6 | 22,527 | 759.6 |

weight, but on inspection, the left kidney of the experimental group was appreciably larger than the controls. These results force the conclusion, that despite increase in size, unilateral nephrectomy during adult life has no effect on the senile atrophy of the opposite kidney as expressed in renal units.

Variations in the size of glomeruli were determined by measuring in Zenker fixed, paraffin sections, the diameters of 200 glomeruli at hilus level, in one adult and one senile kidney. The results are shown in Table II and plotted as frequency curves in Fig. II. The glomeruli in the senile kidney average 8.5 microns larger and show greater

variation in size. If the total surface of the visceral layer of Bowman's capsule is calculated, (Table III) it is found that the loss in renal units is accompanied by an increase in size. That this glomerular enlargement is a true hypertrophy, is not apparent at this time, since there is no evidence that the larger glomerulus has a greater functional capacity. The finding of a greater variation in the size of the glomeruli in senile atrophy is not in harmony with the results of Karsner, Saphir and Todd (7) in cardiac atrophy. In an atrophic heart,

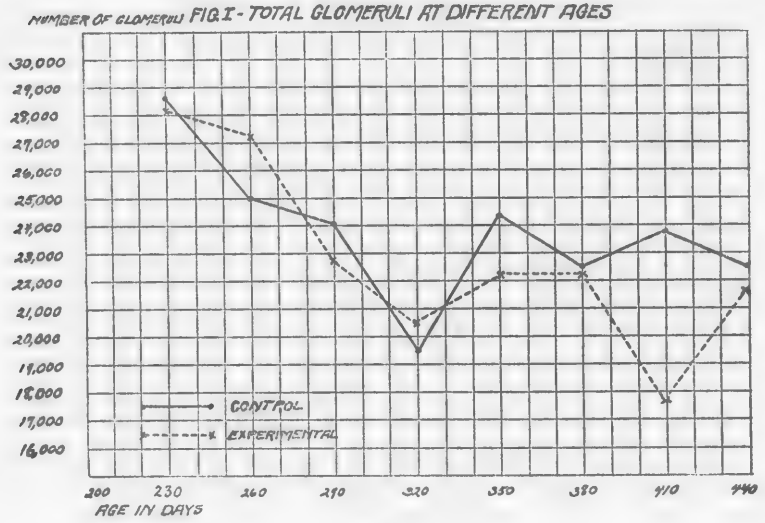


FIG. I

associated with tuberculosis, they found a greater uniformity in the size of muscle fibers than in a normal heart. This variation of morphology in two types of atrophy does not support the hypothesis of Bradley (8) of a single pathogenesis of decreased blood supply.

The method of disappearance of glomeruli in old age is interesting. As yet we have been unable to follow the process. Our present observations are confined to a complete serial section of the right kidney of a rat 440 days of age. In these sections there are insufficient fibrotic glomeruli to account for the total loss and we are inclined to

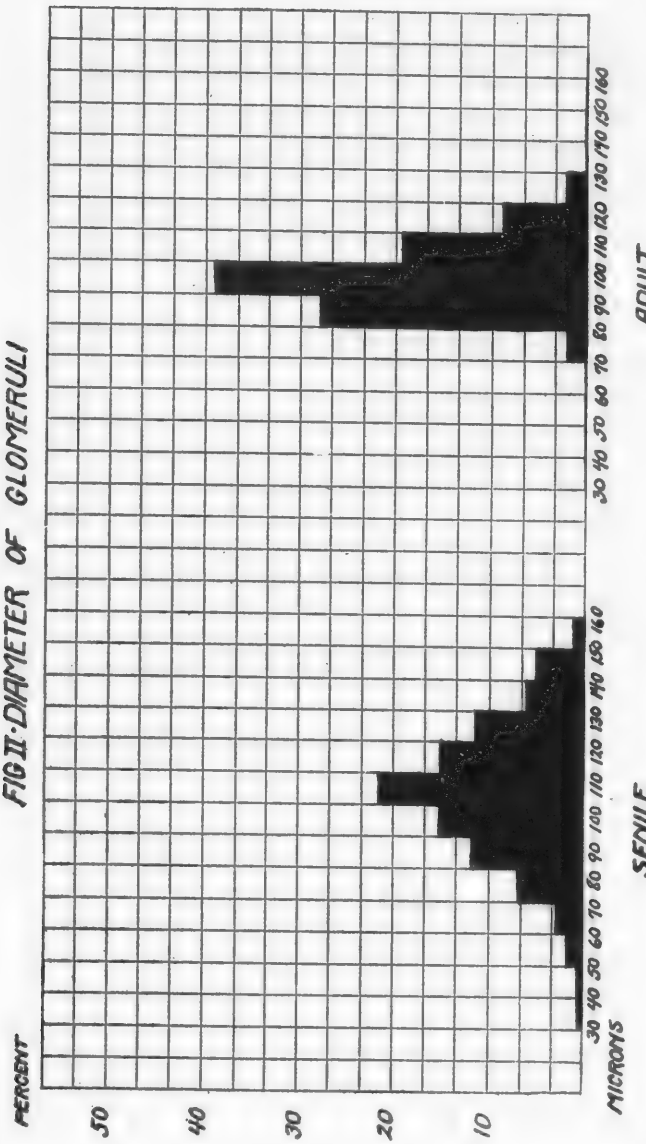
FIG II·DIAMETER OF GLOMERULI
PERCENT
50
40
30
20
10
MICRONS
30 40 50 60 70 80 90 100 110 120 130 140 150 160
SENILE
30 40 50 60 70 80 90 100 110 120 130 140 150 160
ADULT

FIG. II

accept a process of gradual atrophy and complete disappearance as proposed by Chase (6) in Necturus.

The counts on rats No. 4, 14, 15 and 18 are worthy of further comment. The rats designated here as the Yale strain are a mixture of the Wistar rat and an ordinary wild rat. It is evident that this Yale strain is a hybrid stock composed of two substrains, one with 40,000 and the other with 30,000 renal units. This finding together with the possible effect of heredity on the total number of glomeruli is under investigation at the present time.

## DISCUSSION

The results here reported have significance in the interpretation of the remote effects of unilateral nephrectomy in man. The investigation of Hayman and Starr (9) and Moore and Lukianoff (10) have demonstrated that the fluid output of the kidney is dependent in general on the total number of open glomeruli and this latter factor is limited by the total glomerular units in the kidney. If we postulate that approximately 25% of the glomerular filtration space is necessary for basal demands, it follows that the removal of one kidney during adult life leaves a reserve of 100%, but as this same animal approaches senility, further glomerular units are lost and a reserve of only 33% remains. Since the kidney of man undergoes analogous senile changes, it may be safely assumed that a patient under similar circumstances, would develop renal insufficiency after a kidney injury, innocuous to a patient with both kidneys.

The conclusions arrived at in this paper add quantitative support to the doctrine that "old age is inevitable." The senile loss of glomeruli proceeds at the usual rate and to the same extent despite the absence of one kidney. The relations between the total glomerular units and the number of open glomeruli in senile kidneys and the changes in circulation dependent on senility are under investigation in this laboratory.

Arataki (1) found that senility in the Wistar rat occurred after 350 days of age, while our observations place the time at about 300 days. It is possible that senility occurs at different ages in different strains of the same species. Unpublished observations from this laboratory indicate a wide variation in the age period of senile glomerular loss in the human kidney.

## CONCLUSIONS

1. In senility in the white rat there is a decrease in the total number of glomerular units.

2. The decrease in glomerular units is associated with an increase in the average diameter and a greater variation in size, of the remaining glomeruli.

3. Unilateral nephrectomy during adult life has no effect on these senile changes.

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# ON THE ISOLATION, CULTIVATION AND CLASSIFICATION OF THE SO-CALLED INTRACELLULAR "SYMBIONT" OR "RICKETTSIA" OF PERIPLANETA AMERICANA

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PLATE 4

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## 1. INTRODUCTION

The bacteria-like so-called intracellular "symbionts" or "rickettsiae" found in some arthropods have been proven to be transmitted and to be associated with the production of fatal diseases in man and in domestic animals. To the presumably pathogenic forms belong those associated with exanthemic fever, trench fever, Rocky Mountain spotted fever, and heartwater. Dengue fever and some other arthropod-transmitted diseases, such as the Japanese flood fever, are also suspected to be associated with "rickettsiae." One "rickettsia" (*R. melophagi*) is transmitted by its vector (*Melophagus ovinus*) to sheep in which it apparently survives as a harmless parasite. The majority, however, are at present not transmitted to higher animals, but usually exist within certain specialized cells of invertebrates and are passed from one generation to another through their eggs.

Very little is known about either the pathogenic or the non-pathogenic intracellular parasites under discussion because it has so far been impossible to adapt most of them to artificial media. Some claim that the parasites are bacteria (1); others that they should be placed among the recent so-called "rickettsiae" (2) a group erected by da Rocha-Lima in 1916, for certain small microorganisms found in the human body louse infected with the virus of typhus fever. A third class of investigators has concluded that the intracellular inclusions are not microorganisms at all, but products of cellular metabolism (3). Some of the structures observed might represent bacteria; others a totally new type of microorganism; and lastly, still others might, conceivably, represent products of cellular metabolism.

Many arthropods apparently harbor more than one species of "symbiont." In some cases a form pathogenic to vertebrates exists together with a non-pathogenic form. Frequently the two forms resemble one another so closely, morphologically, that cultures become imperative in order to differentiate them, as Noguchi showed in 1926 (4). The cultural possibility also opens up a number

of interesting biological problems among which the much discussed question of symbiosis has prominence.

In 1920 the writer (5) assumed that he had cultivated a spirillum from the bacteriocytes of *Parcoblatta virginica*, and one from *Periplaneta americana* caught near Boston. These spirilla in some of their morphological characters resembled the intracellular parasites of the roaches. However, these results were apparently due to certain technical pitfalls not very evident at that time (6).

### 2. *Location and Morphology of the Intracellular Parasite*

In every individual of *Periplaneta americana*, the large American roach, the bacteriocytes (mycetocytes) are disseminated throughout the fat tissue. Fresh preparations or stained films and sections of fat tissue (at 100 diameters magnification) show the bacteriocytes to be independent, single, irregular cells embedded among fat cell groups. These bacteriocytes possess distinct nuclei and their cytoplasm is packed with granular material. This material resolves itself into definite shapes at magnifications of from 900 to 1800 diameters. Bacteriocytes, excised and triturated in normal salt solution, liberate the cytoplasmic inclusions which appear bacteria-like in form. The elements are not motile and are often congregated into groups. In sections the intracellular units are stained with difficulty. We have obtained satisfactory results by subjecting sections of roaches cut at 8 and 10$\mu$ to a prolonged treatment with Delafield's hematoxylin.

The parasites, liberated by triturating, are very easily stained and are found to be non-encapsulated, Gram-positive and not acid-fast. Smears fixed while wet in alcohol-ether for 15 minutes and stained for 15 to 20 minutes according to Giemsa, give pictures as illustrated in Fig. 1. The microörganisms are pleomorphic, showing equal and unequal division or budding. Two equal or unequal adhering units are common, and three such units are also seen. Coccoidal, short and longer straight or curved bacillary forms are in evidence. These bacilli often terminate in a thickening. The microörganisms may stain uniformly with Giemsa, but frequently assume a banded, granular or bipolar arrangement of the chromatic material. Spores have never been observed. The individual units of the bacillary forms found within the bacteriocytes range in length from 2 to 5$\mu$ with a mean length of 3$\mu$. Their breadth varies from 0.5 to 1$\mu$. Three adhering units may total 8$\mu$ in length and coccoidal or bud forms

measure from 0.5 to 1$\mu$ in diameter. These facts taken into consideration with those previously noted, that the species does not form capsules, is not motile, not acid-fast, but Gram-positive, tentatively place it in the genus *Corynebacterium* (the diphtheroids) of Lehmann and Neumann.

The intracellular parasites are transmitted from generation to generation through the eggs of every individual. The follicular cells of the ovary become infected. Young oöcytes are free from infection, but the more mature ova show a number of bacilli at their surfaces. As the ova grow, the bacteria at their surfaces become more numerous and form a closed layer around the periphery. Still later a definite congregation occurs at each pole. The penetration of the eggs by the parasites occurs late, since the bacteria first actually appear within the eggs after oviposition.

The bacteria are seen in ovarian sections or in films prepared from ova excised from mature females. They are also found within the developing embryos when these are removed from their egg capsules. The parasites found congregated along the periphery of older ova and within developing eggs and embryos are illustrated in Fig. 2. They are slightly more pleomorphic than the bacteriocyte individuals. Short bacilli and coccoidal forms are more numerous than the longer bacilli. The cocci are apparently derived from buds pinched off from longer rods. It appears that the division rate of the parasites is accelerated within the eggs and embryos. In this material the rods have a length range of 0.8 to 4.8$\mu$, with a mean length of 0.9$\mu$.

### 3. *Method Used to Obtain Tissues Free from Contaminants*

We have found the following method satisfactory:

The roach is etherized until immobile. It is then submerged for 15 to 20 minutes in a solution consisting of equal parts of 95 per cent alcohol and 1/1000 mercuric chloride, after which the insect is washed in 70 per cent alcohol. The extremities are amputated at their bases with sterile scissors and the insect is fastened with sterile pins ventral side up in a tray of paraffin recently melted and hardened. Prior to using the paraffin, the tray is flooded with 70 per cent alcohol for a few minutes. After pinning, the ventral side of the abdomen is again washed off with 70 per cent alcohol, and by means of a fine pipette alcohol is forced in between the external abdominal segments. The abdomen is lastly singed with a red hot spatula, and the ventral chitin removed with sterile instru-

ments. Fat or ovarian tissue is then removed from the body cavity and transferred to sterile tubes. Before the tissues are used for cultures, they are macerated with the addition of sterile water. The alimentary tract and any of its adnexa must be avoided.

The female of *Periplaneta americana* lays a hard chitinous egg capsule partitioned in the interior and containing anywhere from 20 to 24 eggs or embryos. Since the eggs and embryos harbor "symbionts" or "rickettsiae," it appeared possible to sterilize the exterior of the capsule, open it, remove the eggs or embryos, macerate them and inoculate media with this material. Before this was accomplished, however, a preliminary microscopic and cultural study seemed necessary. We had no means of knowing whether the interior of the capsule or the partitioned spaces were sterile. Furthermore, the eggs are surrounded with a mucilaginous secretion which might not be sterile. Consequently, freshly deposited capsules were opened and the mucilaginous material examined in the stained state. No microorganisms were revealed. Broth, nutrient agar and dextrose horse blood agar slants and plates were inoculated with the mucilaginous secretion, but subsequently on incubation remained sterile. Macerated eggs and embryos, however, revealed the characteristic diphtheroids. It seems safe to conclude, therefore, that the interior of the normal roach egg capsule is sterile for ordinary bacteria. Normal appearing egg capsules, laid while the insects are in the wild state, are unsafe to use, because some of them are diseased. It is safer to keep captive female roaches under observation during the process of oviposition and take the capsules as soon as they are free.

Capsules so obtained were submerged in the corrosive sublimate-alcohol mixture, previously described, for 30 minutes. They were then washed for 15 minutes in 70 per cent alcohol and rinsed in sterile physiological saline solution. The exteriors of the capsules were tested for sterility by placing them for 4 days in nutrient bouillon and incubating. If the sterilization proved effective the capsules were opened in a small amount of sterile physiological salt solution, the eggs removed and crushed, and the media inoculated.

### 4. *Isolation Experiments*

Attempts were at first confined to transplanting macerated *Periplaneta* ova and bacteriocytes to media at various hydrogen ion concentrations under both aerobic and anaerobic conditions. The results were usually negative. In a modified Noguchi's medium, a suspicious diphtheroidal form was initiated three times and twice an adaptation from this medium to standard nutrient agar was obtained.

*Medium and Methods Adopted.*—The medium and methods finally adopted are as follows:

Standard nutrient agar (10–12 cc.) is poured into petri dishes into which previously 3 cc. of sterile defibrinated horse blood and 2 cc. of a 10 per cent sterile dextrose solution has been added. The final pH value of this medium should be adjusted to a range of from 7.0–7.4. The plates are incubated for 48 hours to test their sterility for ordinary contaminants.

A standard platinum loopful of the macerated ovarian, embryonic or bacteriocyte material, obtained under sterile conditions, is taken and the surface of the medium touched and gently rubbed in an area having a diameter of approximately 0.5 cm. What remains on the loop is then carried over to another area which is gently rubbed in a similar manner. This "spotting technique," as the writer has named it, may be continued without refilling the loop, so that a petri dish may contain anywhere from 2 to 15 or more spots. The number of spots made is entirely arbitrary. After the "spotting," the plates are incubated for 48 hours at 35 or 36°C. They are then examined and it is seen that the spots are dry. If all of the directions are followed very few or no contaminating colonies will develop. When such colonies do appear they are more likely to occur around the edges of the plate and in areas not inoculated. The spots usually appear entirely sterile. Fat drops and other material introduced may persist and give the appearance of minute pinpoint colonies, but actual colonies representing developing microorganisms are difficult to detect. Fishings from the spots are made, nevertheless, and transferred to slants of the same medium as that used in the petri plates. It is important to have a sufficient amount of water of condensation present in the slants. If the quantity appears deficient, a few drops of a solution consisting of 5 cc. bouillon and 1 cc. horse blood serum should be added. In making fishings a loopful of the condensation fluid from a slant is carried over to a spot. The drop is rubbed around on the spot and carried back to the slant and streaked. This procedure is repeated for each spot, so that one finally obtains as many streaked slants as spots present on a plate. The slants are then incubated at 36°C. or are held at room temperature. The tubes should be examined every day after 48 hours and tilted to permit the water of condensation to flood the surface of the medium. A growth may appear anywhere from 48 to 144 hours. At the end of this time, if sterile, the tubes may be discarded. If a growth occurs it is extremely faint and delicate consisting of minute, clear or pale white colonies of pinpoint size.

*Results with Protocols.*—Many unsuccessful attempts were made to obtain primary cultures on slants. Transfers were always prepared from these although at the time no visible growth appeared. However, after the initial development on the plates occurs the microorganisms are easily adapted to the blood agar slants and later to certain other media.

In the first experiment (Table I), two plates represented material from one egg capsule.

Plate 1 was "spotted" 9 times; Plate 2, 6 times. When more than one plate was used the loop was always recharged with the suspension. The plates were incubated 48 hours. At the end of this time, each plate contained two contaminating bacterial colonies, not in close proximity to any of the spots. Fishings were made from all the spots, so that 9 streaked slants represented Plate 1, and 6 Plate 2. These slants were held for 2 days at incubator temperature and for the remainder of the time, up to 144 hours, at room temperature. Slant 3, from Spot 3, Plate 1, yielded a delicate growth in 48 hours. Slant 1, from Spot 1, Plate 2, yielded a similar growth in the same time. These cultures were transferred indefinitely on dextrose blood agar slants and were subsequently classified as Type II. The other "fishings" remained sterile and were discarded.

TABLE I

*Isolation Experiment*

*Egg Capsule*

| From Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | Room T. 72 hrs. | Room T. 96 hrs. | Room T. 120 hrs. | Room T. 144 hrs. | Type |
|---|---|---|---|---|---|---|---|
| 1<br>2 | Sterile | | | | | | |
| 3 | Sterile | +* | + | + | + | + | II |
| 4<br>5<br>6<br>7<br>8<br>9 | Sterile | | | | | | |
| From Plate II Slant No. | | | | | | | |
| 1 | Sterile | + | + | + | + | + | II |
| 2<br>3<br>4<br>5<br>6 | Sterile | | | | | | |

* + signifies growth.

Table II represents an experiment with another egg capsule.

Two plates were "spotted" and 9 spots fished from one, 6 from the other. Plate 1 remained sterile for visible colonies; Plate 2 yielded two contaminants, not near any of the spots. On Slant 1, from Spot 1, Plate 1, a delicate growth was obtained in 144 hours. This growth was subsequently classified as Type III. The remaining tubes proved sterile and were discarded.

TABLE II

*Isolation Experiment*

*Egg Capsule*

| From Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | Room T. 72 hrs. | Room T. 96 hrs. | Room T. 120 hrs. | Room T. 144 hrs. | Type |
|---|---|---|---|---|---|---|---|
| 1 | Sterile | Sterile | Sterile | Sterile | Sterile | + | III |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | | | | | |
| 5 | | | Sterile | | | | |
| 6 | | | | | | | |
| 7 | | | | | | | |
| 8 | | | | | | | |
| 9 | | | | | | | |
| From Plate II Slant No. | | | | | | | |
| 1 | | | | | | | |
| 2 | | | | | | | |
| 3 | | | | | | | |
| 4 | | | Sterile | | | | |
| 5 | | | | | | | |
| 6 | | | | | | | |

Table III represents an experiment performed with fat tissue bacteriocytes and with ova dissected from mature ovaries.

One plate was "spotted" with each and two control plates accompanied the series. These controls were "spotted" with sterile water, incubated, and later "fished." Material from each control spot was transferred to dextrose horse blood agar slants and incubated as in the experiments. Control plates were prepared for all the remaining experiments and all slants from them remained sterile for diphtheroids. Plate 1, from the ova, yielded 4 contaminating colonies.

One contaminant was found on the edge of one spot, but was avoided during the fishing process. Plate 2, from the fat tissue, yielded 2 contaminating colonies, not on any of the spots. Two contaminants, not on spots, developed on each

TABLE III

*Isolation Experiment*

*Fat Tissue and Ova from One Female Roach*

| From Plate I ova Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate I |
|---|---|---|---|---|---|---|---|---|
| 1 | Sterile | Sterile | Sterile | Sterile | + | + | III | |
| 2 | " | " | " | " | + | + | II | |
| 3<br>4 | Sterile | | | | | | | |
| 5 | Sterile | Sterile | Sterile | + | + | + | II | Sterile |
| 6<br>7<br>8 | Sterile | | | | | | | |
| 9 | Sterile | Sterile | Sterile | Sterile | Sterile | + | III | |
| 10<br>11 | Sterile | | | | | | | |
| From Plate II fat tissue Slant No. | | | | | | | | Control plate II |
| 1 | Sterile | | | | | | | |
| 2 | Sterile | Sterile | Sterile | + | + | + | I | |
| 3<br>4 | Sterile | | | | | | | Sterile |
| 5 | Sterile | Sterile | Sterile | Sterile | + | + | I | |
| 6 | " | " | " | " | + | + | II | |

control plate. Slants from the ovarian material yielded Types II and III twice each; slants from the fat tissue bacteriocytes, Type I twice and Type II once. The remaining slants remained sterile.

Table IV represents the result obtained on slants from a small number of fishings from ova and fat tissue from two female roaches.

The fat tissue bacteriocytes yielded Type II, on Slant 2, from Spot 2, Plate 3, Female 1. No contaminating colonies developed on Plate 1 from Roach 1. Plate 2, from the same roach, gave two contaminants not on any of the spots.

TABLE IV

*Isolation Experiment*

*Fat Tissue and Ova from Two Female Roaches*

| Roach I Plate I ova Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate I |
|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | |
| 2 | | | | | | | | |
| 3 | | | | | | | | |
| 4 | | | | | | | | |
| Roach I Plate II ova Slant No. | | | | | | | | Sterile |
| 1 | | | Sterile | | | | | |
| 2 | | | | | | | | |
| 3 | | | | | | | | |
| 4 | | | | | | | | |
| Roach I Plate III fat Slant No. | | | | | | | | Control plate II |
| 1 | | | | | | | | |
| 2 | Sterile | Sterile | + | + | + | + | II | |
| 3 | | | | | | | | |
| Roach II Plate I fat Slant No. | | | | | | | | Sterile |
| 1 | | | Sterile | | | | | |
| 2 | | | | | | | | |
| 3 | | | | | | | | |

Two contaminants, not on the spots, developed on Plate 3 from Roach 1. One contaminant, not on a spot, developed on Plate 1 from Roach 2. No contaminants developed on the two control plates.

An experiment, not tabulated, was performed with fat tissue bacteriocytes from a female roach.

The fishings from the two plates remained sterile. A contaminating colony, not on a spot, developed on each experimental plate; two developed on control Plate 1 and one on control Plate 2.

Table V illustrates the results obtained with fat and ovarian tissue from two females and fat tissue from a male.

TABLE V

*Isolation Experiment*

*Fat Tissue and Ova from Two Female Roaches; Fat Tissue from One Male Roach*

| Roach I Plate I ova Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate I |
|---|---|---|---|---|---|---|---|---|
| 1 | Sterile | Sterile | Sterile | Sterile | Sterile | Sterile | | Sterile |
| 2 | " | + | + | + | + | + | III | |
| Roach I Plate II ♀ fat Slant No. | | | | | | | | Control plate II |
| 1<br>2 | Sterile | | | | | | | Sterile |
| Roach II Plate I ♀ fat Slant No. | | | | | | | | Control plate III |
| 1<br>2<br>3 | Sterile | | | | | | | Sterile |
| Roach III Plate I ♂ fat Slant No. | | | | | | | | Control Plate IV |
| 1 | Sterile | Sterile | Sterile | + | + | + | I | Sterile |
| 2<br>3 | Sterile | | | | | | | |

Plate 1, ovarian material from Roach 1, remained sterile for contaminating colonies. Slant 2 from Spot 2, yielded Type III, however. Two contaminants, not on spots, developed on control Plate 1. Plate 2, fat material from female Roach 1, remained sterile for diphtheroids. One contaminating colony developed in one of the two spots, but was avoided in fishing. Another contaminant was noticed along the edge of the plate. One contaminant developed along the edge of control Plate 2. Plate 1, fat material from female Roach 2, remained sterile

for diphtheroids. Two contaminating colonies developed but were avoided. One was noticed along the periphery of a spot, the other beyond any of the three spots. No contaminants developed on control Plate 3. Plate 1, fat material from male Roach 3, yielded Type I diphtheroid on Slant 1 from Spot 1. Two contaminating colonies, not diphtheroids, developed along the periphery of Spots 2 and 3. These were avoided in fishing and the slants from these two spots remained sterile. No contaminants developed on control Plate 4.

Table VI represents the result obtained with ovarian material from a female roach.

TABLE VI

*Isolation Experiment*

*Ova from a Female Roach*

| Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate |
|---|---|---|---|---|---|---|---|---|
| 1 | Sterile | Sterile | + | + | + | + | I | Sterile |
| 2 | Sterile | | | | | | | |
| 3 | | | | | | | | |
| 4 | | | | | | | | |
| 5 | | | | | | | | |
| 6 | Sterile | Sterile | Sterile | Fungus contamination | Discarded | Discarded | | |
| 7 | Sterile | | | | | | | |
| 8 | | | | | | | | |
| 9 | | | | | | | | |
| 10 | | | | | | | | |
| 11 | | | | | | | | |
| 12 | | | | | | | | |
| 13 | | | | | | | | |
| 14 | | | | | | | | |
| 15 | | | | | | | | |

The fishing from Spot 1 yielded Type I. The rest remained sterile, with the exception of Slant 6 upon the surface of which a fungus developed. Three contaminants grew upon the experimental plate. These were found removed from any of the fifteen spots. Two contaminants grew along the edge of the medium on the control plate.

An experiment, not tabulated, resulted negatively.

One contaminating colony developed in a spot and three others elsewhere. No contaminants developed on the control plate.

Table VII gives the result with fat tissue bacteriocytes from a female roach.

No contaminating colonies developed, but two, beyond the spots, appeared on the control plate. Type II diphtheroid developed on Slant 1 from Spot 1.

TABLE VII

*Isolation Experiment*

*Fat Tissue from a Female Roach*

| Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate |
|---|---|---|---|---|---|---|---|---|
| 1 | Sterile | Sterile | + | + | + | + | II | Sterile |
| 2 | Sterile | | | | | | | |
| 3 | | | | | | | | |
| 4 | | | | | | | | |
| 5 | | | | | | | | |
| 6 | | | | | | | | |
| 7 | | | | | | | | |
| 8 | | | | | | | | |
| 9 | | | | | | | | |
| 10 | | | | | | | | |
| 11 | | | | | | | | |

TABLE VIII

*Isolation Experiment*

*Fat Tissue from a Male Roach*

| Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate |
|---|---|---|---|---|---|---|---|---|
| 1 | Sterile | | | | | | | Sterile |
| 2 | | | | | | | | |
| 3 | Sterile | Sterile | Sterile | + | + | + | II | |
| 4 | Sterile | | | | | | | |
| 5 | | | | | | | | |
| 6 | | | | | | | | |
| 7 | | | | | | | | |
| 8 | | | | | | | | |
| 9 | | | | | | | | |
| 10 | | | | | | | | |

Table VIII shows that Type II on Slant 3 from Spot 3 was the only significant result.

Three contaminants were obtained on the experimental plate but they grew outside of the spots. Two contaminants, outside of the spots, appeared on the control plate.

The experiment given in Table IX yielded Type I on Slants 3 and 11, and Type III on Slants 9 and 10.

Three contaminating colonies beyond the spots, developed on each plate, both in the experiment and in the control.

TABLE IX

*Isolation Experiment*

*Fat Tissue from a Female Roach*

| Plate I Slant No. | 36°C. 24 hrs. | 36°C. 48 hrs. | 36°C. 72 hrs. | 36°C. 96 hrs. | 36°C. 120 hrs. | 36°C. 144 hrs. | Type | Control plate |
|---|---|---|---|---|---|---|---|---|
| 1<br>2 | Sterile | | | | | | | Sterile |
| 3 | Sterile | + | + | + | + | + | I | |
| 4<br>5<br>6<br>7<br>8 | Sterile | | | | | | | |
| 9 | Sterile | + | + | + | + | + | III | |
| 10 | " | + | + | + | + | + | III | |
| 11 | " | + | + | + | + | + | I | |
| 12<br>13<br>14 | Sterile | | | | | | | |

*Analysis of the Isolation Experiments.*—Fishings were made from the spots on the plates after 48 hours although no macroscopic growth was visible. At first the incubation of the plates was prolonged 5 days, but this had no greater advantage than the 2 day incubation. Stained films direct from the spots, after 24 hours, showed many degenerating and Gram-negative diphtheroids. A few remained normal and retained the gentian violet. These few Gram-positive rods seemed physiologically active and were in the process of adaptation

to their new environment. The first conditions appeared necessary to initiate this adaptation but were not sufficient to bring its realization to complete fruition. When transplanted to the second environment (dextrose horse blood agar slants) further development proceeded and small colonies were formed. Further adaptation resulted during successive transplants. Altogether 138 spots were made and 20 cultures obtained from these. Approximately 14 per cent of the attempts succeeded.

The time consumed between the plate "fishings" and the appearance of a growth on the slants varied. Seven cultures were macroscopically observed in 48 hours, three in 72 hours, four in 96 hours, four in 120 hours, and two in 144 hours. No further cultures were obtained after 144 hours.

The first few spots on the plates, in general, yielded a greater percentage of slant cultures than the last. This was probably due to progressive dilution. The fact remains that six cultures were obtained from the first spots, four from the second, three from the third, two from the fifth, one from the sixth, two from the ninth, one from the tenth, and one from the eleventh.

In regard to the three types, Types I and III were each recovered 6 times and Type II, 8 times. The three types were recovered from females, Types I and II from males, and Types II and III from capsules. This does not signify that Type III does not occur in males, nor Type I in capsules. The data on these points are too small. One individual usually yielded only one type, but in one case the three types were recovered from one female, and Types I and III were recovered from another. Types II and III were recovered from ova and embryos, and Types I and II from mature fat tissue bacteriocytes. The number of times each type was recovered from the ova is practically identical with the frequency with which the same type was recovered from the mature bacteriocytes.

The few contaminations encountered on many of the experimental and control plates were derived from the atmosphere during the hardening of the media. After pouring the plate, the cover was permitted to remain partly open for a few minutes in order to allow the water vapor to escape, as is customarily done. This procedure may have been the principal source of the contaminants, but they usually developed at a considerable distance from where the material was planted

and were avoided during the fishing process. Moreover it is signficant that no control plate yielded diphtheroidal microorganisms.

Many unsuccessful attempts were made to isolate diphtheroidal microorganisms from the alimentary tract, from the feces and from the exterior of *Periplaneta*. Recently, however, one spot from one plate out of four inoculated with a fecal emulsion yielded a form resembling Type III. The intracellular parasites undoubtedly often gain an entrance into the digestive system due to the omnivorous and filthy feeding habits of roaches. The many unsuccessful attempts to isolate the microorganism from this source, however, seem to show that few remain viable after ingestion.

### 5. *Morphological, Cultural, and Biological Characters of the Three Types*

The three types cultivated from *Periplaneta* are Gram-positive, not acid-fast, not motile, non-sporulating and non-capsulating. All have a tendency to clump. When 48 hour cultures on dextrose horse blood again are fixed while wet in alcohol-ether for 15 minutes and stained

TABLE X

*Measurements of the Periplaneta Parasites, Compared with Those of the Three Cultured Types*

| Source of parasites | Length range | Mean length | Breadth range | Diameter range of coccoidal forms |
|---|---|---|---|---|
| | μ | μ | μ | μ |
| Roach bacteriocytes | 2–5 | 3 | 0.5–1 | 0.5–1 |
| Roach ova | 0.8–4.8 | 0.9 | 0.5–1 | 0.5–1 |
| Culture Type I | 0.5–3.2 | 1.95 | 0.4–0.6 | 0.5–1 |
| Culture Type II | 1.6–4.8 | 3.49 | 0.5–1 | 0.5–1 |
| Culture Type III | 1.6–3.2 | 2.12 | 0.4–0.6 | 0.3–0.8 |

according to Giemsa, they show the following characters illustrated in the figures. Type I, Fig. 3, is a moderately thin banded or bipolar bacillus with a few oval or coccoidal forms. The bacilli are straight, slightly curved or crescent-shaped. Type II, Fig. 4, is a longer and thicker banded bacillus showing racket or club-shaped terminations. These bacilli are also usually flexed and at times form chains of two or three units. Oval and coccoidal forms are also present and appear to be derived from terminal buds formed by the bacilli. Equal and unequal division appears to occur. Type III, Fig. 5, represents an

extremely pleomorphic type, a coccobacillus or a rickettsia form. Banded straight or slightly curved bacilli are present but the oval and especially the coccoidal forms dominate. By comparing the cultural forms with the forms obtained directly from *Periplaneta* (Figs. 1 and 2), differences are seen but these are fundamentally slight. The morphological characters of cultures are the most reliable ones which can be used for differentiating the three types.

Differentiation of the three types on a cultural basis is not possible. However, slight differences as they consistently appeared, immediately after isolation, will be given. Growth on the best medium, dextrose horse blood agar, is weak, but by successive transfers the microörganism was gradually adapted to certain routine media. Practically all of the cultural characters reached their peak in 120 hours at 36°C.

In standard nutrient bouillon, no clouding appeared up to 8 days. A slight, viscid sediment was formed at that time. Microscopical examination in 120 hours demonstrated a weak growth of the three types, with a tendency to agglutination. Nutrient bouillon containing 20 per cent horse serum and 1 per cent dextrose showed moderate clouding in 120 hours for Type I. Types II and III did not cloud this medium although a microscopical examination revealed growth. Moderate growth was obtained for all three types on Dorsett's egg medium. Milk seemed a poor medium; no coagulation was obtained in one month, although at that time the milk had a slightly acid reaction. Potato yielded a poor growth. Nutrient agar slants after 120 hours showed a weak growth consisting of a few clear, round, minute, pinpoint colonies. Coagulated horse serum slants yielded a slightly heavier growth of the same character as on the nutrient agar. Dextrose horse blood agar slants showed a good, although delicate growth in 120 hours. In 8 days the pinpoint colonies became pale white in color. Horse blood agar slants, without dextrose, yielded a weaker growth. Gelatin stabs held at 22°C. in 8 days showed a very weak growth, beaded along the line of puncture. In 2 months no liquefaction occurred. Dextrose horse blood agar plates gave clear, round or irregularly round, pinpont colonies in 120 hours which later assumed a pale white color. For Type I these colonies measured 1/3 to 1/2 mm. in diameter. For Types II and III the colonies measured up to 3/4 mm. in diameter reaching 1 mm. in diameter in 8 days. Types I and II colonies also possessed a slightly raised center. Growth was inhibited on nutrient agar plates. On 1 per cent dextrose agar plates a few colonies appeared which had the same appearance as those on dextrose horse blood agar.

Carbohydrate fermentation tests did not yield any differential characters. The three types do not produce any gas; they ferment glucose, sucrose and maltose, but not lactose or mannite. Since horse serum stimulated the growth of the microorganisms slightly, 1 per cent of each carbohydrate was added to

nutrient bouillon containing 20 per cent of horse serum. Before adding the maltose to the serum bouillon, the latter was heated at 60°C. for 30 minutes (7).

The maximum acidity, reached in some cases in 5 days, in others not before 17 days, is recorded in Table XI.

TABLE XI

| Carbohydrates | Glucose | Lactose | Sucrose | Maltose | Mannite |
|---|---|---|---|---|---|
| pH of medium | 7.3 | 7.3 | 7.3 | 7.3 | 7.3 |
| " " Type I | 5.2 | 7.3 | 5.2 | 5.3 | 7.4 |
| " " " II | 5.2 | 7.3 | 5.0 | 5.3 | 7.4 |
| " " " III | 5.4 | 7.2 | 5.6 | 5.3 | 7.3 |

Agglutination tests were undertaken to determine, if possible, a further qualitative or quantitative difference between the three types. Some diphtheroids do not form antibodies readily, so a prolonged and varied treatment of rabbits is often necessary.

Rabbits at first received 13 intraperitoneal inoculations, at suitable intervals, of 1.5 cc. of living microorganisms derived from cultures gradually adapted to nutrient agar slants and upon the surface of which good, but delicate 120 hour growths were obtained. The entire 120 hour growth from a slant was suspended in 2 cc. of physiological salt solution and used for each inoculation. Three intraperitoneal inoculations with 2 cc. followed. The rabbits were then bled from their ears, but the titer was still low. Three intraperitoneal inoculations again followed, given on three consecutive days, 0.5 cc. on the 1st, 1 cc. on the 2nd, and 2 cc. on the 3rd day. The rabbits were then rested for 10 days, after which they received intravenous inoculations on 3 consecutive days, 1 cc. on the 1st, 1.5 cc. on the 2nd, and 2 cc. on the 3rd. One week following this procedure blood serum was again obtained.

Table XII gives the tests performed with suspensions of nutrient agar cultures 120 hours old of the three types against normal and immune rabbit sera. Due to the tendency to natural clumping which the three types exhibit, all suspensions were thoroughly shaken prior to performing the tests.

A marked distinction exists between the three types. Spontaneous complete agglutination at all dilutions occurred with Type II and normal serum as well as with salt solution, but since this never occurred with the other two types it must be accepted as a difference.

Table XIII shows a series of agglutination tests with the three types against their own immune sera and against the immune sera of

TABLE XII

*Agglutination Tests*

Normal rabbit sera and immune rabbit sera against Types I, II, and III. Suspensions of microorganisms in 0.2% NaCl solution. Standardization on Gates turbidity scale to 2.4.

| | Serum dilutions | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10240 | 1/20480 | 1/40960 | 1/81920 | NaCl |
| Normal serum Type I | − | − | − | − | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± |
| Immune serum I Type I | +++ | +++ | +++ | +++ | ++ | ± | ± | ± | ± | ± | ± | ± | ± | ± | |
| Normal serum Type II | C | C | C | C | C | C | C | C | C | C | C | C | C | C | C |
| Immune serum II Type II | C | C | C | C | C | C | C | C | C | C | C | C | C | C | |
| Normal serum Type III | ++ | ++ | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± |
| Immune serum III Type III | C | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | ± | ± | |

TABLE XIII

*Reciprocal Agglutination Tests*

| Immune serum | I | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum dilutions | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10240 | 1/20480 | 1/40960 | 1/81920 |
| Type I | +++ | +++ | +++ | +++ | ++ | ± | ± | ± | ± | ± | ± | ± | ± | ± |
| " II | C | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| " III | C | C | C | C | C | C | C | ++ | + | ± | ± | ± | ± | ± |

| Immune serum | II | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum dilutions | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10240 | 1/20480 | 1/40960 | 1/81920 |
| Type I | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| " II | C | C | C | C | C | C | C | C | C | C | C | C | C | C |
| " III | + | + | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± | ± |

| Immune serum | III | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serum dilutions | 1/10 | 1/20 | 1/40 | 1/80 | 1/160 | 1/320 | 1/640 | 1/1280 | 1/2560 | 1/5120 | 1/10240 | 1/20480 | 1/40960 | 1/81920 |
| Type I | ± | ± | − | − | − | − | − | − | − | − | − | − | − | − |
| " II | C | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| " III | C | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | + | + | ± |

the others. Marked differences are noticeable with a tendency for Type II to agglutinate strongly at all dilutions and with each of the sera.

### 6. *Filtration Experiment*

Filtrable stages of certain rickettsiae have been reported in the literature. Since the three types cultivated by us are rather pleomorphic, especially Type III, it was important to determine whether stages exist small enough to pass the pores of diatomaceous filters.

Dextrose blood agar slant cultures, 120 hours old, of the three types were separately suspended in 10 cc. physiological salt solution. These solutions were filtered through Berkefeld grade "N" candles at a pressure of 74 mm. of mercury. The time of filtration and the amount of the filtrates obtained were constant. Each filtrate was then centrifuged for 30 minutes at 1,000 R.P.M., after which 0.1 cc. of the upper liquid and 0.1 cc. of the lower portion were added to the surface of dextrose blood agar slants. All of the tubes so inoculated remained sterile after an incubation of 2 weeks, showing that nothing passed the filters.

### 7. *Classification*

The sizes and shapes of the three types of microörganisms studied correspond to those of the intracellular parasites of *Periplaneta americana*. The intracellular and cultivated forms are straight or curved rods with a tendency to club and racket shapes. All are pleomorphic and show barred, uneven staining. They are not acid-fast, but Gram-positive and non-motile. The cultured forms are aerobic, and since insect tissue is primarily aerobic (8), it may be assumed that the intracellular forms are adapted to oxygen. No endospores occur in the parasitic or cultured forms. These considerations, primarily, automatically place the species within the genus *Corynebacterium* (the diphtheroids). For the species we would like to propose the name *periplanetae*, and the variety studied by us may be known as *americana*, including three types. The term *Corynebacterium periplanetae* n. sp. variety *americana* has the following advantages: The generic name places the microörganism among the diphtheroidal bacilli. The specific name associates the species with the insect genus *periplaneta* and the variety name with the insect species *americana* and not with another species, as for example *Periplaneta australasiae*. Should it be later shown that all members of the genus *periplaneta* harbor the

identical species of intracellular parasite the variety name could be discarded.

8. *General Immunity of Periplaneta towards Its Intracellular Inhabitants and towards the Cultured Types*

To prove further the identity of the cultured species with the intracellular parasite, we attempted agglutination tests with the immune rabbit sera against the diphtheroids obtained from the insect fat tissue and ova. It was extremely difficult, however, to obtain heavy enough suspensions of the microorganisms from these sources. Extraneous material, such as tissue fragments, fat, etc., interfered with the reactions and with the reading of the data. Complement fixation tests were tried, but the technical difficulties which the material presented were too great and these methods were abandoned. Another approach, however, itself. The speculation which led to the experiment follows.

Roaches must exhibit a general immunity towards their intracellular parasites; otherwise these would not be restricted to certain prescribed cells, but would grow uninhibited anywhere in the body. If the cultured and intracellular forms were inoculated into the body cavity of normal roaches they should be inhibited or destroyed provided the host is sensitized to both; in other words, provided both microorganisms are members of the same species. On the other hand, bacteria foreign to the body of the roaches as a rule kill them when they gain access to the body cavity (9). Some experiments support the above speculations.

Two mature roaches received intraperitoneal inoculations of 0.1 cc. of a 10 cc. physiological salt solution suspension of a 24 hour agar culture of a *Bacillus coli* from calves; two the same dose of *Staphylococcus pyogenes albus*, two the same dose of *Staphylococcus muscae* (10), and two a culture of a bacillus isolated by the writer from *Stomoxys calcitrans*. Death of all these roaches occurred between 24 and 72 hours after infection. Cultures and films from blood and tissues showed many of the inoculated bacteria. Following this test six roaches were inoculated intraperitoneally with 0.2 cc. of 1 cc. physiological salt solution suspension of the intracellular parasites obtained from fat cell bacteriocytes. None were present in the blood 24 hours after inoculation, and the six roaches remained alive. Four hours after inoculation blood films stained with Wright's stain

revealed a few degenerated pink stained diphtheroids. The same result was obtained by inoculating each of the three cultivated organisms intraperitoneally into six roaches.

The above experiments indicate that *Periplaneta americana* is sensitized to both the parasitic and cultured forms, but is not so sensitized to other bacteria.

## 9. SUMMARY AND CONCLUSIONS

In *Periplaneta americana*, the large American roach, bacteriocytes are found in both sexes. These bacteriocytes are scattered throughout the fat tissue and their cytoplasm is filled with microorganisms. Evidence is presented to show that the intracellular forms are diphtheroidal bacilli. These diphtheroids are transmitted from one generation to another through the ova.

A method is described whereby tissues, free from contaminants, may be obtained from *Periplaneta*. A medium and a new method, the "spotting technique," are described, by means of which initial cultures of the parasites were obtained from eggs within capsules, from ova and fat tissue bacteriocytes. Two conditions appear necessary, one to initiate adaptation to the new environment, the other to bring its complete realization to fruition. When development has been properly initiated, further adaptation occurs with successive transfers until finally certain other media appear suitable. Approximately 14 per cent of the isolation and cultivation attempts succeeded. All of the isolations were studied and it was found that three morphologically distinct types had been cultivated. In general one host yielded only one type, but one female revealed three types and another two. One type was also isolated once from a fecal emulsion. The probable reason for this result is discussed.

The three types isolated were diphtheroidal bacilli resembling one another closely enough to be considered a single species but invariably offering distinct minor differences to warrant a separation into three distinct types. These three types have remained true to their original forms and sizes through 52 transfers. The sizes, general morphology, and tinctorial reactions of the three types cultivated correspond to

the intracellular parasites of *Periplaneta americana*. The cultural and biochemical activities of the strains did not reveal any sound characters for differentiating types. Serologically, however, useful distinctions were found.

Some additional evidence along immunological lines is offered to show that the microorganism cultivated is a representative of the identical species parasitic within *Periplaneta americana*.

The evidence appears sound to the writer that the *Periplaneta* parasite has been isolated and cultivated over 20 times and that it is a bacterium belonging to the genus *Corynebacterium* (the diphtheroids). For the species the name *Corynebacterium periplanetae* nov. sp. variety *americana* is proposed.

The three cultural types did not produce forms small enough to pass through the pores of Berkefeld "N" candles.

The writer wishes to acknowledge the interest and able assistance given by Mr. N. A. Coria during the course of this work.

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## EXPLANATION OF PLATE 4

Sketches drawn at 1800 diameter magnification. Alcohol-ether fixation. Stained according to the method of Giemsa.

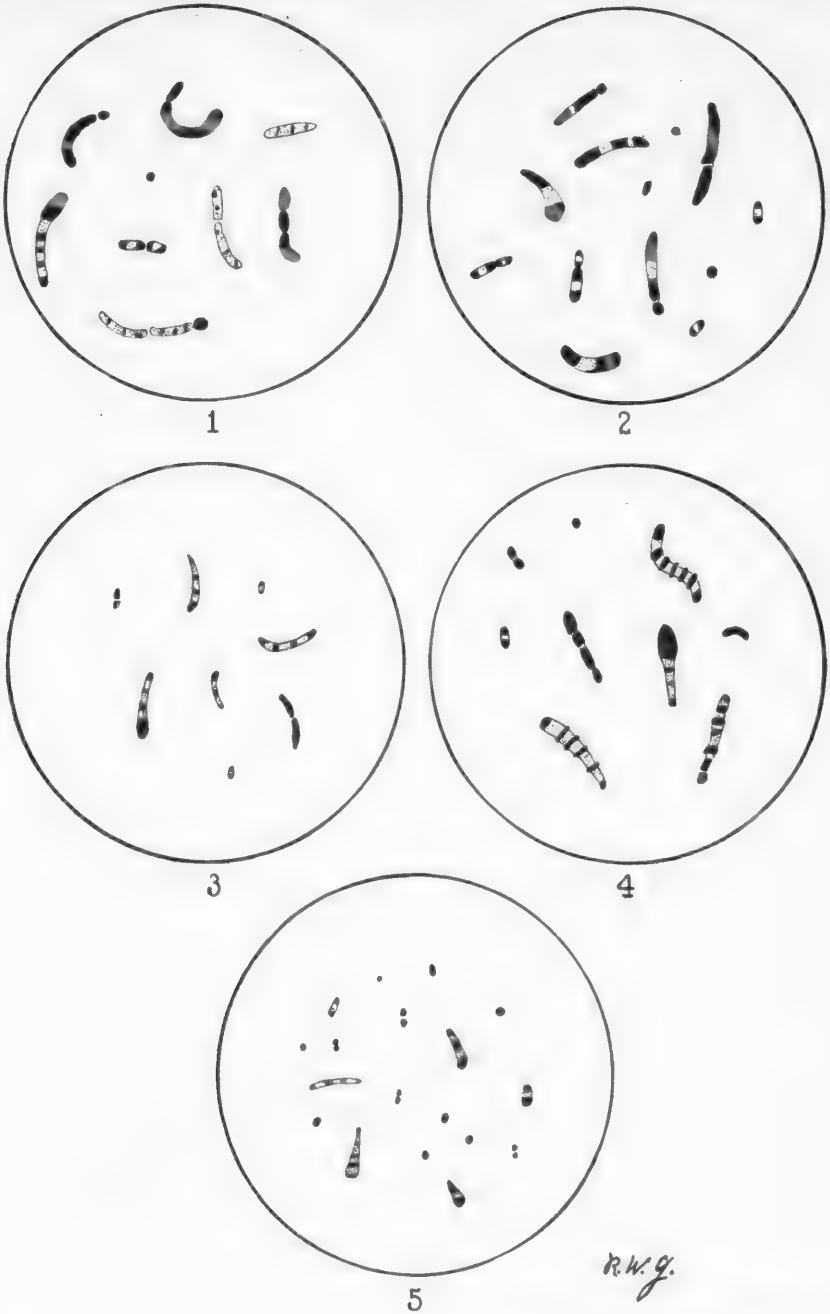
FIG. 1. *Corynebacterium periplanetae* var. *americana* from fat tissue bacteriocytes of *Periplaneta americana*.

FIG. 2. The same parasite from the ova of *Periplaneta*.

FIG. 3. Type I. 48 hour culture of the same microorganism on dextrose blood agar.

FIG. 4. Type II. 48 hour culture of the same microorganism on same medium.

FIG. 5. Type III. 48 hour culture of same microorganism on same medium.




(Glaser: "Rickettsia" of *Periplaneta americana*)

# STUDIES IN THE BLOOD CYTOLOGY OF THE RABBIT

## I. Blood Counts in Normal Rabbits

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The cells of the peripheral blood in rabbits have been studied in this laboratory in connection with the problem of the constitutional factors related to the occurrence and course of disease, and with especial reference to the influence of environmental conditions (1). Although many investigations have dealt directly or indirectly with the cytology of rabbit blood comparatively little has been published regarding the blood cell picture of normal rabbits from the standpoint of a large material. Furthermore, the possibility of spontaneous variations over periods of time with respect to general environmental conditions such as seasonal change has received but scant attention. These features are among those included in the present study. Observations have also been made on various types of animal material and in connection with studies primarily concerned with factors of an environmental (experimental) nature. Still other observations have been carried out on rabbits inoculated with *Treponema pallidum*, a malignant tumor, and virus III, the first two agents being those with which most of our studies on constitutional and environmental factors have been conducted.

The results of the investigation will be published in a series of papers of which this is the first. It contains the results of a statistical analysis of 1110 blood counts obtained from 174 normal rabbits, with especial attention directed to distribution frequencies. The values obtained from this analysis will be used in subsequent papers as a basis with which to compare the results of other experiments. Preliminary notes on certain phases of the study have already been published (2).

## *Materials and Methods*

The rabbits employed were, as far as could be determined, a fair sample of the animal material available for experimental purposes. For the most part, the ordinary brown and gray types predominated but the type described as the Flemish cross or mixture was also represented, and there were a few black and albino animals. Only male rabbits approximately 6 to 8 months old were used; a few were thought to be slightly younger and a few were probably a month or two older. Each rabbit was caged separately in a well lighted (sunlight), well ventilated room; the diet throughout the period of observation consisted of hay, oats, and cabbage.

The results reported are based upon 1110 blood counts on 174 rabbits during 13 months, from October 20, 1927 to November 22, 1928. The majority of examinations, 1001 in number, were made during the period ending June 20, 1928. The observations were derived from 3 sources of animal material:

I. 10 rabbits; 426 observations. Between October 20, 1927 and June 20, 1928, blood counts on 5 animals were made at weekly intervals. With the other 5 rabbits, biweekly examinations were carried out from October 20, 1927 to February 10, 1928 and thereafter to June 19, 1928 at weekly intervals.

II. 10 rabbits; 130 observations. Weekly counts were made from March 29, to June 19, 1928.

III. 154 rabbits; 554 observations. This mixed group contains, first, single observations on 9 groups of 5 to 10 rabbits each, a total of 75 animals, examined from December 16, 1927 to August 9, 1928, and within a few days from the time of receipt from the dealers. Second, there are 470 observations distributed among 11 groups of 5 to 10 rabbits each, a total of 70 animals; these groups were examined 4 to 11 times during 1 to 8 weeks. Third, a single observation on 9 of the 10 rabbits comprising group I made 4 months after the regular examinations had been discontinued, is included in this group.

A uniform routine with respect to the time and the method of collection and examination of the blood was followed. The rabbits were fed as usual on the day preceding the examination but received no food on that day until after the specimens of blood had been obtained. The majority of observations were made from 9 A.M. to 1 P.M. In the case of 2 groups with a large number of observations, the interval at which examinations were made was largely determined by other experiments in progress; the rabbits were always examined in the same order on the same day of the week and at the same or approximately the same hour.

The blood was obtained from an ear vein without disturbance by simply placing the rabbit upon a rough surface such as a towel stretched tightly across a table top. An assistant supported the head and held the ear forward from its base in a convenient position. The external surface of the ear was moistened with dilute alcohol and a small area was carefully shaved. The shaved skin was dried with gauze and the vessels dilated by means of an electric bulb beneath the ear. A small vessel was punctured with the point of a corneal knife and a few drops of blood allowed to flow freely before any was collected. The specimens for the following

examinations were then obtained in the order designated: the differential white count (supravital technic), the white cell count, the hemoglobin estimation, and the red cell count. All specimens from 4 rabbits were taken one after the other and the counts were then made immediately. The method of Sabin (3) was followed for the supravital preparations, vital neutral red dye[1] being used and 100 white cells in each specimen counted. Standardized pipettes and the usual dilutions of 1:100 of Hayem's solution for the red cells and 1:10 of a 1.0 per cent acetic acid solution for the white cells were employed. The hemoglobin content was determined by the Newcomer hemoglobinometer, using 0.2 cc. blood in 10 cc. of $\frac{N}{10}$ hydrochloric acid.

In the analysis of results, absolute numbers of cells per cmm. of blood have been considered. The distribution curves given in Text-figures 1 to 8 are based upon the use of a group interval of approximately one-tenth of the mean value of each class of cells. The curves obtained by plotting the actual results were smoothed by the formula $\frac{a+2b+c}{4}$; the initial value of each smoothed curve is represented by $\frac{2b+c}{3}$ and the final value by $\frac{a+2b}{3}$. In some instances, the curves are not extended to the upper limits but the values omitted are few and scattered. The limit to which a curve should be extended is indicated by a figure placed at the termination of the curve.

The mode as reported was obtained by the use of King's formula applied to the unsmoothed distribution curve, mode $= 1 + \frac{f2}{f2+f1}c$, in which 1 is the lower limit of the group in which the greatest number of values is massed, $f$ 2 the frequency in the group above, and $f$ 1 the frequency in the group below the modal group, and $c$ the size of the group interval. By varying $f$ 1 and $f$ 2 to represent the combined frequencies of the 2 or 3 groups below and above the modal group, the formula was in reality applied to a smoothed curve. In the case of the hemoglobin in which the modal group is approached by an adjoining group, $c$ represents 2 groups. The empirical formula of Pearson, mode = mean − 3 (mean − median), and the mean of the modal group were also used to check the results of the above method.

The figures as given include all data. No count has been omitted because of the occurrence of such conditions as snuffles or ear canker in occasional rabbits observed for the longer periods. In all other cases, the animals were apparently free from disease. It seemed best to present the material in the present form be-

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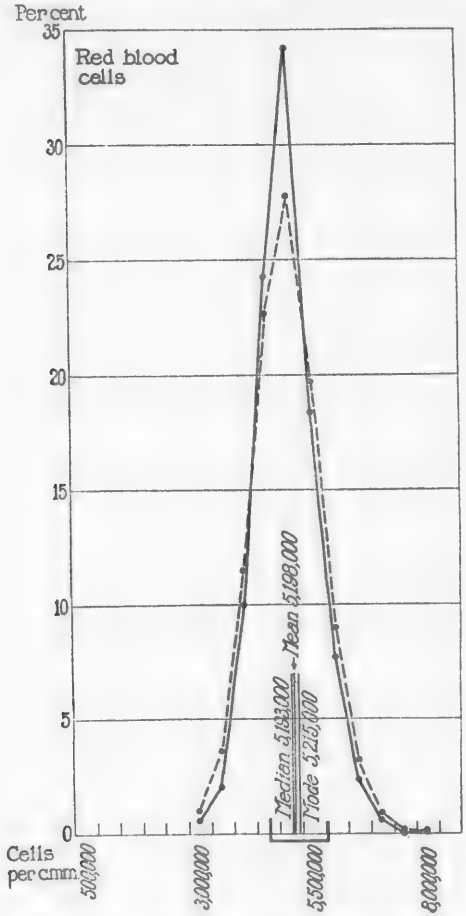
[1] The dye employed was neutral red iodide No. 2 supplied through the kindness of Dr. Barnett Cohen, Hygienic Laboratory, Washington, D. C.

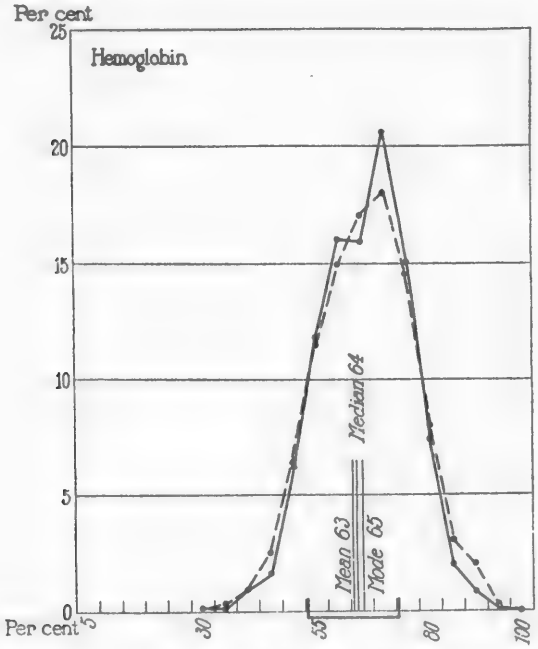
fore attempting to make corrections for disease or any other factor that might be associated with variations in the numbers of cells.




TEXT-FIG. 1. Distribution frequency of numbers of red blood cells per cmm. in per cent. In this and subsequent text-figures, the smoothed curve is graphed as a broken line and the brackets on the base line denote the limits of the standard deviation.

RESULTS

The results obtained in this series of 1110 blood cell determinations on 174 normal rabbits are presented in condensed form in Table I and Text-figures 1 to 8. The analysis of this material has included the determination of the following numerical values for the total red and white cells and the several classes of white cells per cmm., and the hemoglobin content: minimum, maximum, and mean counts, the probable error of the mean, the mode, the median, the standard deviation and the coefficient of variation (Table I). The distribution frequencies of the cells and the hemoglobin have also been determined (Text-figs. 1 to 8).

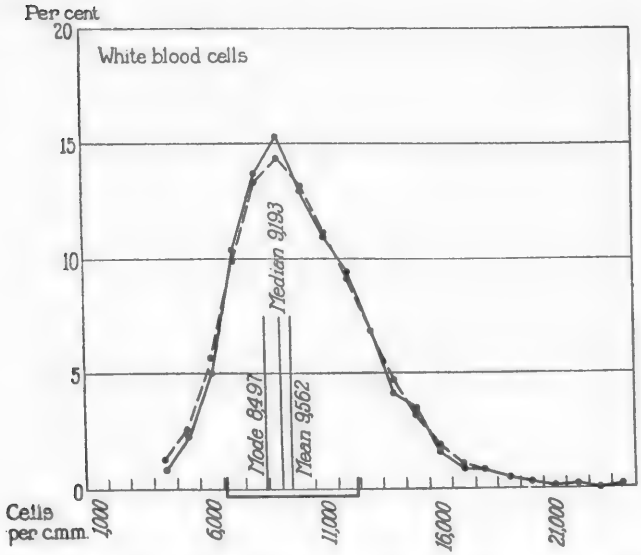



TEXT-FIG. 2. Distribution frequency of percentage of hemoglobin content in per cent.

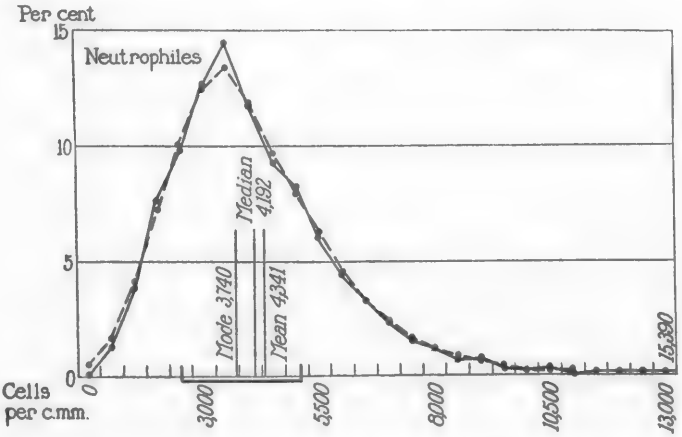
TABLE I

*Summary of Numerical Values Obtained from 1110 Blood Counts on 174 Male Rabbits of Various Ages and Breeds—from October 20, 1927 to November 21, 1928*

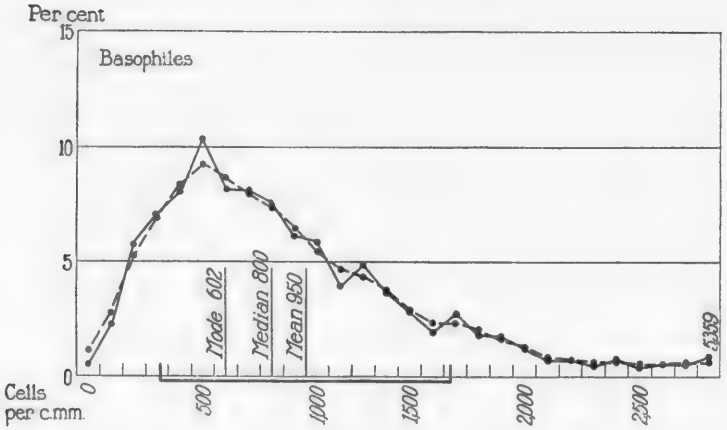
| | Mean | Mode | Median | Minimum | Maximum | Standard deviation | Coefficient of variation |
|---|---|---|---|---|---|---|---|
| | *per cmm.* | *per cmm.* | *per cmm.* | *per cmm.* | *per cmm.* | *per cmm.* | *per cent* |
| Red blood cells | 5,198,000 ±12,700 | 5,215,000 | 5,193,000 | 3,020,000 | 8,040,000 | 628,250 | 12.09 |
| Hemoglobin | 63% ± 2% | 65% | 64% | 28% | 90% | 10% | 15.87 |
| White blood cells | 9562 ± 59 | 8497 | 9193 | 3150 | 23500 | 2919 | 30.53 |
| Neutrophiles | 4341 ± 37 | 3741 | 4192 | 1050 | 15390 | 1823 | 41.99 |
| Basophiles | 950 ± 13 | 602 | 800 | 0 | 5359 | 635 | 66.84 |
| Eosinophiles | 214 ± 4 | 90 | 159 | 0 | 1760 | 217 | 101.40 |
| Lymphocytes | 3045 ± 28 | 2402 | 2904 | 630 | 9900 | 1366 | 44.86 |
| Monocytes | 1000 ± 12 | 750 | 898 | 72 | 5405 | 571 | 57.10 |



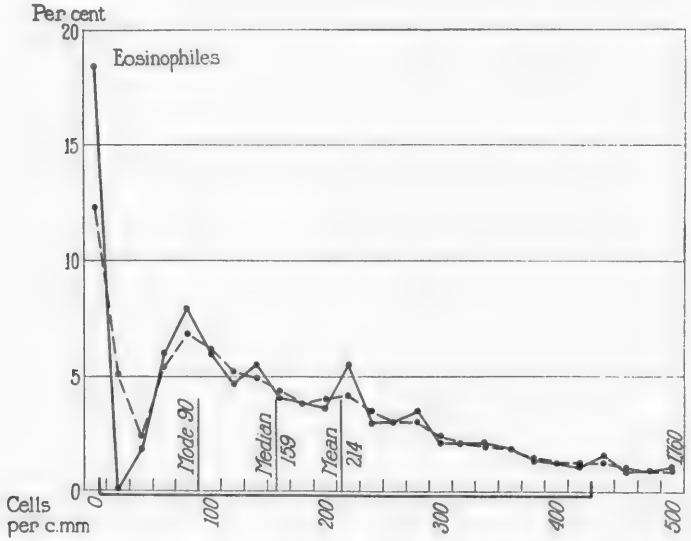

TEXT-FIG. 3. Distribution frequency of total numbers of white blood cells per cmm. in per cent.



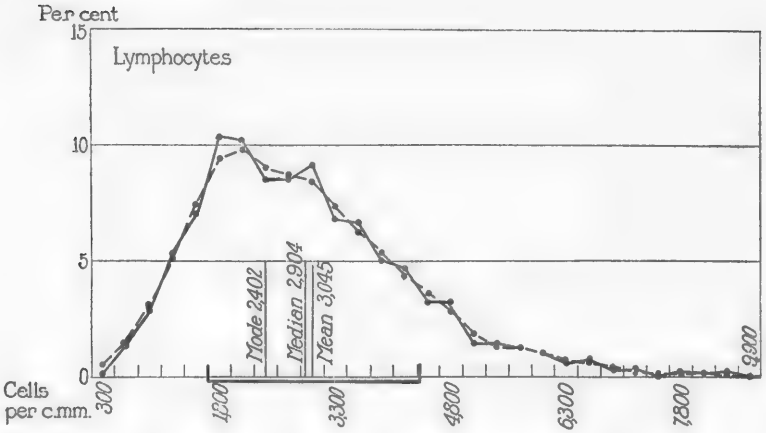

TEXT-FIG. 4. Distribution frequency of numbers of neutrophiles per cmm. in per cent.



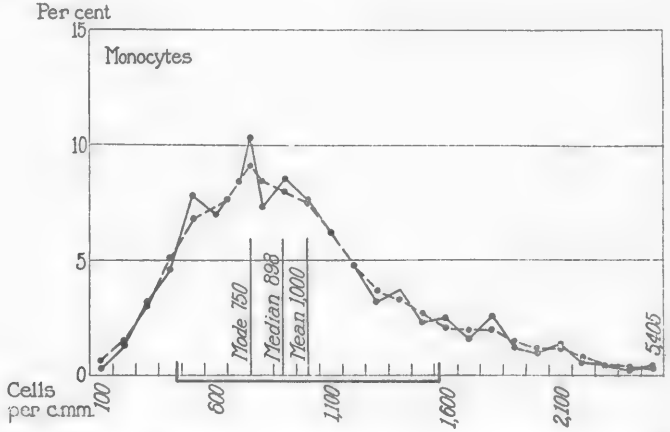

TEXT-FIG. 5. Distribution frequency of numbers of basophiles per cmm. in per cent.




TEXT-FIG. 6. Distribution frequency of numbers of eosinophiles per cmm. in per cent.




TEXT-FIG. 7. Distribution frequency of numbers of lymphocytes per cmm. in per cent.




TEXT-FIG. 8. Distribution frequency of numbers of monocytes per cmm. in per cent.

## DISCUSSION AND CONCLUSIONS

In discussing the results of these experiments, it should be borne in mind that no effort was made to maintain uniformity of material other than that the rabbits employed were comparable with those used in other experiments. And the same may be said regarding the conditions of living while the animals were under observation. Various breeds of rabbits were used, certain groups were studied for short and others for long periods of time, while other series were examined but once. These conditions, of course, tend to emphasize the element of variability. One purpose of this study, however, was to ascertain the possible as well as the probable limits of variation in the numbers of cells and in the hemoglobin content. In the present paper, the results are considered primarily from the standpoint of the range of individual counts rather than from that of the various groups of rabbits comprising the entire series.

An examination of the data contained in Table I and in the distribution curves of Text-figs. 1 to 8 shows that with one exception, the numbers of cells in the peripheral blood of rabbits are subject to wide variations. The exception is the erthrocytes, the coefficient of variation of which is 12.09 per cent, a value much lower than those of the various granulocytes, the lymphocytes, and the monocytes. As might be expected in view of this finding, the variation of the hemoglobin content is of a similar order of magnitude, its coefficient of variation being 15.87 per cent. In contrast to these findings, the various white cells are characterized by a high order of variability. The coefficients of variation of the neutrophilic leucocytes and the lymphocytes are 41.99 and 44.86 per cent; those of the basophiles and monocytes are somewhat higher, 66.84 and 57.1 per cent while that of the eosinophiles reaches the high value of 101.4 per cent. The variability of the total white blood cells, on the other hand, occupies a lower level, its coefficient of variation being 30.53 per cent.

It will be seen that the distribution curves for the red blood cells (Text-fig. 1) and the hemoglobin content (Text-fig. 2) have the same general form and are almost symmetrical; they show a relatively narrow range of numerical distribution with a rapid rate of increase and decrease in the percentage of counts whose numbers are close to the mean and mode and have but a slight tendency to prolongation at either

extreme. Both curves, however, show a slight left skew, a feature which is brought out more clearly by the relative positions of the mean, the median, and the mode. The differences between the values for the mean and mode are extremely small, 17,000 cells for the erythrocytes and 2 per cent for the hemoglobin respectively. It is not unlikely that with a larger number of observations the curves would conform to theoretical expectations. In the case of the hemoglobin, the curve in its ascending portion at the 60–65 per cent level shows a peculiar flattening which is reflected in the more rounded shape of the ascending as compared with the descending limb of the smoothed curve. The explanation of this peculiar feature is not entirely clear but it may be a question of the matching of shades at this particular level in making the readings.

The distribution frequencies for the other cells are much more irregular. With the single exception of the eosinophiles (Text-fig. 6), the total white cells, the neutrophiles, the basophiles, the lymphocytes, and the monocytes (Text-figs. 3, 4, 5, 7, 8) have a tendency to follow the same general form of numerical distribution as the red blood cells and the hemoglobin content but the curves are of a much wider range and are decidedly skewed to the right, due to the occurrence of varying counts at levels well above the mean. The form of the curves for the total white cells and the neutrophiles shows that the most frequent values occupy a considerably narrower range than those of the basophiles, lymphocytes, and monocytes. In the case of these 3 classes of cells, the highest portion of the curves shows a fairly wide plateau, the right extreme of which corresponds approximately to the level of the mean while the left falls below the mode. The relative positions of the mode, the median, and the mean conform to theoretical expectations in all cases.

The curves for the total white blood cells and the neutrophiles are quite regular but those of the other classes of white cells show many irregularities chiefly in their descending portions. These conditions extend the zone of high frequency distribution and in addition, are responsible for the high coefficients of variation. The distribution curve for the eosinophiles (Text-fig. 6) differs from all the others with respect to the high level of its initiation, but if this portion of the curve is omitted, its general form resembles the others. It is, however,

somewhat flatter. The peculiar first part of the curve is due to the fact that in many counts no eosinophiles were seen. In order to make the chance of including a fair representation of eosinophiles equal to that of the monocytes and basophiles for example, it would be necessary to count 500 instead of 100 cells upon the basis of the ratio of the respective mean values for these cells which is approximately 5:1 (Table I). As with the other classes of white cells, the curve for the eosinophiles shows a marked skew to the right indicating a wide range of high frequencies. Its irregularities (descending portion), however, are more pronounced than those of the other cells with the possible exception of the monocytes. These conditions make for the high order of magnitude—101.4 per cent—of the coefficient of variation.

The curves for the total white cells and the neutrophiles are very similar and at first glance, it might be supposed that the distribution of neutrophile values was responsible for this similarity. But the neutrophiles represent approximately only half the total number of white cells (Table I) and since the coefficient of variation of the total white cells is less than that of the neutrophiles, it is evident that the other cells, considered collectively, would form a distribution curve not unlike that of the neutrophiles and with a smaller coefficient of variation than those of the individual classes of cells. This would suggest that while these various classes of cells vary widely, they tend to preserve some kind of a relation so that their total numbers, from a collective point of view, are approximately as constant as the numbers of neutrophiles.

The results of this study with respect to the mean values for the red and white blood cells and for the neutrophiles are of the same general order as those reported by others, as for example Bushnell and Bangs (4), Scott and Simon (5), and Cunningham, Sabin, Sugiyama, and Kindwall (6) (Table II). With the other classes of white cells, however, there is less agreement except in the case of the last named authors whose mean values for the lymphocytes and monocytes differ but little from those here reported. It should be noted that the differential counts of Bushnell and Bangs, and presumably those of Scott and Simon, were made from fixed preparations while Cunningham, Sabin, Sugiyama, and Kindwall used the supravital technic.

TABLE II

*Comparison of Blood Cell Values as Given by Various Authors*

| | *Number of counts* | *Number of rabbits* |
|---|---|---|
| Bushnell and Bangs | 100 | 100 |
| Scott and Simon | 100 | 100 |
| Cunningham et al. | 217* | 53 |
| Pearce and Casey | 1110 | 174 |

| | Mean | Standard deviation | Coefficient of variation | Percentage of white blood cells |
|---|---|---|---|---|
| | *per cmm.* | *per cmm.* | *per cent* | *per cent* |
| Red blood cells | | | | |
| Bushnell and Bangs | 5,989,500 | 779,358 | 13.01 | |
| Pearce and Casey | 5,198,000 | 628,000 | 12.09 | |
| White blood cells | | | | |
| Bushnell and Bangs | 10,675 | 2224 | 20.83 | |
| Scott and Simon | 11,105 | | | |
| Cunningham et al. | 11,281 | | | |
| Pearce and Casey | 9,562 | 2919 | 30.53 | |
| Neutrophiles | | | | |
| Bushnell and Bangs | 4174 | 1161 | 27.57 | 39.1 |
| Scott and Simon | 3825 | | | 34.4 |
| Pearce and Casey | 4341 | 1823 | 41.99 | 45.4 |
| Basophiles | | | | |
| Bushnell and Bangs | 382 | 232 | 60.61 | 3.4 |
| Scott and Simon | 135 | | | 1.2 |
| Pearce and Casey | 950 | 635 | 66.84 | 9.9 |
| Eosinophiles | | | | |
| Bushnell and Bangs | 120 | 90 | 75.00 | 1.1 |
| Scott and Simon | 374 | | | 3.3 |
| Pearce and Casey | 214 | 217 | 101.40 | 2.2 |
| Lymphocytes | | | | |
| Bushnell and Bangs | 5754† | 1196 | 20.77 | 53.9 |
| Scott and Simon | 6297 | | | 56.7 |
| Cunningham et al. | 2805 | | | 24.9 |
| Pearce and Casey | 3045 | 1366 | 44.86 | 31.8 |
| Large mononuclears | | | | |
| Bushnell and Bangs | 49 | 54 | 118.60 | 0.43 |
| Scott and Simon | 532 | | | 4.7 |
| Transitionals | | | | |
| Bushnell and Bangs | 114 | 96 | 84.11 | 1.07 |
| Monocytes | | | | |
| Cunningham et al. | 943 | | | 8.43 |
| Pearce and Casey | 1000 | 571 | 57.1 | 10.5 |

* This value which does not appear in their publication, was supplied by the authors.

† Small lymphocytes only.

In regard to the coefficient of variation, the values of Bushnell and Bangs and those reported here are in close agreement for the red blood cells but with the other cells the differences range from 6.23 in the case of the basophiles to 26.4 for the eosinophiles. Similar differences are also found in the standard deviation values of the two series. In view of the experimental and technical circumstances, however, it is not surprising that the results of several series of observations, such as those contained in Table II, do not agree more closely. The relative numbers of counts and of animals employed, the method used for the differential white cell counts, and the nature of the animal material are among the factors which obviously affect the nature of results of this character.

Although the present study is based upon a large number of observations, a comparatively large number of normal stock rabbits and an acceptable method of white cell differentiation, the values submitted for the total red and white cells counts, for the numbers of the various classes of white cells, and for the hemoglobin content in the peripheral blood should be accepted as approximate values of normality. They serve the useful purpose of orientation and in addition, may be employed as a basis of comparison for the results of other experiments. But it will become evident when the various small groups comprising the series and the factors affecting them are considered, that the influence of existing conditions must be taken into account before any value be accepted as a standard of normality.

## SUMMARY

A study of the blood cytology of normal male rabbits was carried out from October 20, 1927 to November 22, 1928 in connection with an investigation of constitutional and environmental factors related to the occurrence and course of disease. In 1110 observations on 174 animals, total red and white cells counts, differential white cell counts by the supravital method, and hemoglobin estimations were made.

A statistical analysis of the results obtained is presented. Attention is directed to the occurrence of wide variations in the numbers of the various white cells as contrasted with comparatively small variations in the numbers of red cells and of hemoglobin content.

The results recorded are regarded as representing approximate rather than fixed values of normality.

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# THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

## I. The Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type*

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In previous communications (1, 2) it was shown that avirulent, non-type-specific R forms of Pneumococcus may be converted into virulent, type-specific, S organisms either "*in vivo*" by animal passage, or "*in vitro*" by growth in anti-R serum. Conversion by both methods was invariably accompanied by the acquisition of all the characteristics of the S type, including maximal virulence. In every instance in which the change was effected by these procedures the R forms were converted to the same specific type from which they were originally derived.

Since the publication of the foregoing results there has appeared a most significant article by F. Griffith (3), in which he stated that:

1) R forms of Pneumococcus may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2) R forms of Pneumococcus may be similarly converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures.

3) R forms of Pneumococcus may be transformed into S forms of heterologous types by the subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of heterologous S cultures.

The present communication is concerned with the first two of the

* In this and succeeding instances 'homologous type' indicates that specific S type from which the R forms were originally derived.

above findings. The third finding, which involves the question of actual transformation of type, is the subject of the succeeding paper.

## *A. Conversion of R Forms of Pneumococcus into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Large Amounts of Living R Organisms*

### *Methods*

Ten-hour plain broth cultures of Pneumococcus were centrifuged and the bacteria were resuspended in plain broth in varying dilutions as outlined in the following experiments. Mice were inoculated subcutaneously in the right inguinal region, with 0.5 cc. of each of the various dilutions, care being taken that none of the material escaped along the track of the needle. All mice were autopsied in the following manner: The skin was washed with alcohol and a subcutaneous incision was made along the mid-line of the abdomen. A flap of skin was then reflected and cultures were made from the site of injectioon on blood agar plates and in blood broth. The inguinal gland, or a portion of tissue from this region, was carefully excised under sterile precautions and cultures were made from this material in blood broth. Except in earlier experiments contaminations rarely occurred. Occasionally mice developed ulcers at the site of injection and such animals were discarded. Cultures were made from the heart's blood in the usual manner.

### EXPERIMENTAL

(*a*) *2 R culture, strain D/39/R.*

This culture was obtained from a typical Type II S Pneumococcus by growth in homologous immune serum. Its virulence was such that 0.5 cc. of culture occasionally killed white mice, but amounts of 0.25 cc. or less uniformly failed to do so. It produced only Rough colonies, did not agglutinate specifically in type sera, and did not produce the specific soluble substance upon which type-specificity depends (4). It could be changed to the S type by either the "*in vivo*" method of animal passage, or by the "*in vitro*" method of growth in media containing anti-R serum. Three to four mouse passages usually sufficed to bring about the R→ S change, while five to seven transfers in 10 per cent anti-R serum induced a similar transformation. Single-cell cultures derived from the mass culture have been shown to react in precisely the same manner.

A series of eight mice were injected subcutaneously in the right inguinal region with the bacteria from varying amounts of culture, a volume of 0.5 cc. being injected in each instance.

TABLE I

| No. mice | Amount of living R forms injected—in terms of original culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|
| | | | P.I. | H.B. | |
| | *cc.* | | | | |
| 2 | 10 | 1 d. 40 hrs. | R and S | S | Type II |
| | | 2 " " " | " " " | " | " " |
| 2 | 5 | 1 d. 18 hrs. | R only | R only | R |
| | | 2 " 40 " | R and S | S | Type II |
| 2 | 2.5 | 1 d. 40 hrs. | " " " | " | " " |
| | | 2 " 72 " | " " " | " | " " |
| 2 | 1 | 1 d. 30 hrs. | " " " | " | " " |
| | | 2 " 48 " | " " " | " | " " |

d; died.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. mice injected, 8.
Died, 8. Reversion to the homologous S type 7.
No reversions, only R organisms recovered, 1.

In seven out of eight animals injected, type-specific organisms, possessing all the attributes of the S type, were recovered from the heart's blood. Cultures from the site of injection yielded a mixture of R and S colonies. The one animal yielding only R forms died in 18 hours, apparently before there was time for the transformation to be effected.

(*b*) *3 R culture, strain M/3/R.*

This culture was obtained from a typical Type III S Pneumococcus by growth in homologous immune serum. It possessed all the characteristics of the R form and could be converted to the S type, although not so readily as the 2 R culture above described. Twenty to thirty animal passages by the intraperitoneal route were necessary to restore type-specificity, while ten to fifteen transfers in 10 per cent anti-R serum were required to effect the R→S change.

Ten mice were injected subcutaneously as follows:—

TABLE II

| No. mice | Amount of living R forms injected—in terms of original culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|
| | | | P.I. | H.B. | |
| | *cc.* | | | | |
| 2 | 30 | 1 d. 18 hrs.<br>2 " 48 " | R only<br>R and S | R only<br>S | R<br>Type III |
| 2 | 15 | 1 d. 48 hrs.<br>2 " 64 " | R and S<br>" " " | S<br>S | " "<br>" " |
| 2 | 10 | 1 d. 18 hrs.<br>2 " 100 " | R only<br>R and S | R only<br>S | R<br>Type III |
| 2 | 5 | 1 d. 40 hrs.<br>2 s. | R and S<br>— | S<br>— | " "<br>— |
| 2 | 2.5 | 1 s.<br>2 s. | —<br>— | —<br>— | —<br>— |

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. mice injected, 10.
Died, 7. Reversion to the homologous S type, 5.
No reversion, only R organisms recovered, 2.
Survived. 3.

While this culture reverted to the homologous S type in five out of ten animals injected, larger doses were required than with the 2 R culture described. This finding is in accord with the results obtained by the other methods of inducing reversion.

(*c*) *1 R culture, strain 1/192/R.*

This R culture had been under artificial cultivation in this laboratory for many years. All previous efforts to effect reversion to the S type had been unsuccessful. Reimann (5) passed the same strain through 105 consecutive mice without altering its characteristics and 100 transfers in 10 per cent anti-R serum likewise induced no change.

Nine mice were injected subcutaneously, each with the bacteria from 50 cc. of R culture, as follows:

TABLE III

| No. mice | Amount of living R forms injected—in terms of original culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|
| | | | P.I. | H.B. | |
| | *cc.* | | | | |
| 9 | 50 | 1 d. 18 hrs. | R only | R only | R |
| | | 2 " " " | " " | " " | " |
| | | 3 " " " | " " | " " | " |
| | | 4 " " " | " " | " " | " |
| | | 5 " 21 " | " " | " " | " |
| | | 6 " " " | " " | " " | " |
| | | 7 " 60 " | " " | " " | " |
| | | 8 " 80 " | " " | " " | " |
| | | 9 s. | — | — | — |

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No mice injected, 9.
Died, 8. Reversion to the homologous type, O.
No reversion, only R organisms recovered 8.
Survived, 1.

In spite of the large amounts of organisms employed,—the bacteria from 50 cc. of culture—this strain, in all animals, failed to revert to the S type. Smaller amounts of culture were used in other experiments with uniformly negative results. This finding offers further evidence of the existence of different degrees of constancy in the R form of Pneumococcus and confirms the results previously obtained.

## *Conversion of Single-Cell R Cultures into S Forms of the Homologous Type*

In a previous paper (2) it was reported that single-cell cultures have always reacted in the same manner as the mass cultures from which they were obtained. To substantiate this finding single-cell R strains were selected from the above mass cultures, according to the method of Avery and Leland (6), and injected subcutaneously into white mice. In all cases essentially the same results were obtained as when the

mass cultures were used. The following protocol shows the results of a typical experiment:

*Single-cell strain, 2 R culture, strain D/39/R.*

TABLE IV

| No mice | Amount of living R forms injected—in terms of original culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|
| | | | P.I. | H.B. | |
| | cc. | | | | |
| 2 | 10 | 1 d. 24 hrs.<br>2 " 40 " | R only<br>R and S | R only<br>S | R<br>Type II |
| 2 | 5 | 1 d. 22 hrs.<br>2 " 24 " | R only<br>" " | R only<br>" " | R<br>R |
| 2 | 2.5 | 1 d. 54 hrs.<br>2 s. | R and S<br>— | S<br>— | Type II<br>— |
| 2 | 1 | 1 d. 40 hrs.<br>2 s. | R and S<br>— | S<br>— | Type II<br>— |

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. mice injected, 8.
Died, 6. Reversion to the homologous S type, 3.
No reversion, only R organisms recovered, 3.
Survived, 2.

In this experiment three mice apparently died prematurely of an R infection. Otherwise the results were essentially the same as those recorded in Table I in which the mass culture was employed.

### *Attempts to Cause Further "Degradation" of an R Culture by Prolonged Growth in Homologous Immune Serum*

In the course of later work it became essential to have a 2 R culture which would not revert so readily to the S type. Accordingly the above 2 R strain was grown in 50 per cent Type II serum for twelve further transfers and the resulting culture injected into mice as follows:—

TABLE V

| No. mice | Amount of living R forms injected—in terms of original culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|
| | | | P.I. | H.B. | |
| | cc. | | | | |
| 2 | 10 | 1 d. 21 hrs.<br>2 " 40 " | R only<br>R and S | R only<br>S | R<br>Type II |
| 2 | 5 | 1 d. 40 hrs.<br>2 " " " | R and S<br>" " " | S<br>" | Type II<br>" " |
| 2 | 2.5 | 1 d. 54 hrs.<br>2 s. | R and S<br>— | S<br>— | Type II<br>— |
| 2 | 1 | 1 d. 70 hrs.<br>2 s. | R and S<br>— | S<br>— | Type II<br>— |

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. mice injected, 8.
Died, 6. Reversion to the homologous S type, 5.
No reversion, only R organisms recovered, 1.
Survived, 2.

By comparison with the results in Table IV it is seen that, in spite of twelve further transfers in 50 per cent homologous immune serum, this strain reverted to the homologous S type as readily as the original culture.

It would appear that an R culture possesses the capacity to become "stabilized" at some phase of the "degradation" process. In subsequent experiments evidence will be offered to show that a corresponding condition of partial degradation may occur in S cultures. In other words, the terms R and S, as applied to cultures of Pneumococcus, have only a relative value.

The results of the preceding experiments may be summarized as follows:—R forms of Pneumococcus can be converted into S forms of the homologous type by the subcutaneous injection in white mice, of suitable amounts of R cultures. As in other methods, conversion was

invariably accompanied by the acquisition of all the attributes of the S type, including maximal virulence. Single-cell cultures reacted in the same manner as the mass cultures from which they were derived. Attempts to cause a further "degradation" of R forms by continued growth in homologous immune serum were unsuccessful.

### *B. Conversion of R Forms of Pneumococci into S Forms of the Homologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of the Homologous "S" Culture*

#### *Methods*

It is of paramount importance to consider in detail the methods employed in the production of the vaccines[1] and the controls adopted to eliminate the possibility of the persistence of viable forms in the suspensions of heat-killed organisms. All vaccines were made from 1500 cc. of plain broth cultures grown in 3 liter flasks. In earlier experiments little attention was paid to the phase of growth at which the culture was killed, or to the amount of autolysis which might have taken place at the time of heating. In later experiments, however, these factors were found to be of considerable importance, and ten-hour plain broth cultures were uniformly used. Moderately heavy growth was found to be essential. After ten hours growth at 37°C. the flasks of culture were subjected to a *preliminary* heating at 60° for 10 minutes. This preliminary heating inhibited further autolysis and was found to have considerable influence on the efficacy of the vaccine. The cultures were concentrated by centrifuging and taken up in 1/100th of their original volume of plain broth. This concentrated material was transferred to glass ampules, sealed in a blow flame, and heated when totally immersed in water. The vaccines were subjected to definite temperatures for varying periods, as will later be described. Since the factors of time and heat materially altered the results obtained they were most carefully regulated. Fifteen minutes was the shortest period and 60°C. the lowest temperature to which the bacterial suspensions were exposed, and invariably this minimal exposure was found sufficient to kill all pneumococci. In many experiments, however, the vaccines were heated at much higher temperatures and for longer periods of time.

Mice were injected subcutaneously in the right inguinal region, the total volume introduced never exceeding 0.75 cc. Under these conditions the development of ulcers at the site of injection did not occur except in occasional instances. The

[1] For the sake of convenience the term vaccine is used to denote a suspension of heat-killed organisms.

animals were autopsied as described in the first part of this paper. Contaminations were rarely encountered. Cultures were invariably made, both from the site of injection and the heart's blood, on blood agar plates and in blood broth. The colonies were examined under a Zeiss "plate culture" microscope; but morphology alone was never considered a final criterion as to the nature of the organisms constituting the colony. Agglutination tests were done on all cultures. In cases of doubt a second mouse was inoculated and the organisms from the peritoneal contents were typed in the usual manner.

*Controls on the Viability of the Vaccines:*

The possibility of potentially viable organisms surviving in the concentrated vaccines demanded that more than ordinary control measures should be adopted to eliminate such a contingency. The "*in vitro*" and "*in vivo*" controls employed were as follows:

*1. In Vitro Controls:*

(a) Cultures were made from all vaccines in blood broth and on blood agar plates. In many experiments this was done in varying dilutions. In no instance was growth obtained.

(b) Many lots of S vaccine were used repeatedly in "*in vitro*" attempts to secure the R→ S change. Broth containing concentrated S vaccine was seeded with R forms and subcultured serially for twenty transfers. No growth of "S" organisms occurred and the final culture remained avirulent for mice.

(c) In one critical experiment cultures were made, both aerobically and anaerobically, in 5 per cent blood broth and blood-extract dextrose broth. The cultures were incubated two weeks, plates poured, and the material injected into mice. No growth occurred and the mice survived.

*2. "In Vivo" Controls:*

(a) Control mice were injected with the vaccine alone. At least four mice were always used and in many experiments the number of control animals was equal to the number of experimental animals. Varying amounts of vaccine up to and including the bacteria from 100 cc. of culture were injected. Both the subcutaneous and intraperitoneal routes were used. Without exception all animals survived. They were sacrificed at intervals up to three weeks and autopsied. The inguinal lymph gland, or a portion of subcutaneous tissue at the site of injection, was dissected out and cultures were made from this material in blood broth and on blood agar plates. In some cases this tissue was ground up and injected into other mice. Cultures were also invariably made from the heart's blood. In no instance were living pneumococci recovered.

(b) Control mice were injected with the vaccine together with other live organisms. The possibility of the existence in the vaccines of potentially viable organisms, which could not multiply by themselves, but which might, in some way, be stimulated to renewed growth by other live organisms, received careful consideration. Mice were injected with vaccines together with living cultures of Staphylococcus, Streptococcus, B. Influenzae, and Friedländer's bacillus. All

animals which succumbed were autopsied and all surviving animals were sacrificed at appropriate intervals and careful cultures were made. In no instance was a viable pneumococcus recovered.

As Griffith pointed out (3), in certain instances, the temperature at which the vaccines were heated exerted a definite influence on the effect which they produced. The following experiments are therefore divided into two groups, 1) those in which the vaccines were heated at 60°C, and 2) those in which the vaccines were heated at 100°C. In both groups the time of heating was fifteen minutes.

### *I. R Cultures Together with Homologous S Vaccines, Heated for 15′ at 60°C.*

*(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15′ at 60°C.*

TABLE VI

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 7 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 1½ days | R only | R only | R |
| | | | 2 " " " | R and S | S | Type I |
| | | | 3 " 5 " | S | S | " " |
| | | | 4 " 7 " | S | S | " " |
| | | | 5 s. k. 7 " | R only | — | — |
| | | | 6 s. k. 7 " | R only | — | — |

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 4. Reversion to the homologous S type, 3.
No reversion, only R organisms recovered, 1.
Survived, 2. R organisms recovered when sacrificed, 2.

(*b*) *2 R Culture* (*Strain D/39/R*) + *II S Vaccine, Heated for 15′ at 60°C.*

TABLE VII

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in colonies | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 7 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 1½ days | R and S | S | Type II |
| | | | 2 " " " | " " " | S | " " |
| | | | 3 " 2 " | " " " | S | " " |
| | | | 4 " " " | " " " | S | " " |
| | | | 5 " " " | " " " | S | " " |
| | | | 6 s. ulcer at P.I. | — | — | — |

d; died.
s; survived.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 5. Reversion to the homologous S type, 5.
Survived, 1. (Ulcer).

*(c) 3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15′ at 60°C.*

TABLE VIII

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in colonies | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 7 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 2 days | R and S | S | Type III |
| | | | 2 " " " | " " " | " | " " |
| | | | 3 " 2½ " | " " " | " | " " |
| | | | 4 " 3 " | " " " | " | " " |
| | | | 5 " 4 " | " " " | " | " " |
| | | | 6 s. k. 11 " | R only | — | — |

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 5. Reversion to the homologous S type, 5.
Survived, 1. R organisms recovered when sacrificed, 1.

The results of the three preceding experiments may be summarized as follows; R forms of Pneumococcus, when injected subcutaneously in white mice, together with S vaccines of the homologous type, were converted, in the majority of the animals, to the specific S type from which they were originally derived. In these experiments the vaccines were heated for 15′ at 60°C. All control mice survived. At the end of seven days the controls were sacrificed and cultures were made from both the site of injection and the heart's blood. Without exception all cultures were sterile. The 1 R culture (Strain 1/192/R) which had remained totally avirulent after all previous efforts to effect the R→S change, reverted to the S type in three out of six animals injected.

## *II. R Cultures Together with Homologous S Vaccines, Heated for 15′ at 100°C.*

### *(a) 1 R Culture (Strain 1/192/R) + 1 S Vaccine, Heated for 15′ at 100°C.*

TABLE IX

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 7 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 1½ days | R only | R only | R |
| | | | 2 " 2 " | R only | R only | R |
| | | | 3 s. | — | — | — |
| | | | 4 s. | — | — | — |
| | | | 5 s. k. 9 days | R only | — | — |
| | | | 6 s. | — | — | — |

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 2. Reversion to the homologous S type, 0.
No reversion, only R organisms recovered, 2.
Survived, 4. R organisms recovered when sacrificed, 1.

Since reversion to the S type failed to occur in any of the mice which received Type I S vaccine heated at 100°, while the change was frequently effected in those which received the vaccine heated at 60°C., this experiment was repeated. Entirely similar results were obtained. Apparently, as reported in Griffith's paper, Type I vaccine, heated at 100°C. fails to produce the change brought about by the vaccine heated at 60°C.

Attention is drawn to the fact, that, in some mice, living R forms

were found in the subcutaneous tissues nine days after injection. In other experiments they have been recovered as late as twenty days after inoculation. The ability of R forms to survive in the tissues, then, is not the only condition necessary to bring about conversion to the S type.

(*b*) *2 R Culture (Strain D/39/R) + II S Vaccine, Heated for 15′ at 100°C.*

TABLE X

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in colonies | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 9 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 1½ days | R and S | S | Type II |
| | | | 2 " " " | " " " | " | " " |
| | | | 3 " 2 " | " " " | " | " " |
| | | | 4 " " " | " " " | " | " " |
| | | | 5 " " " | " " " | " | " " |
| | | | 6 " " " | " " " | " | " " |

d; died.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 6. Reversion to the homologous S type, 6.

(*c*) *3 R Culture (Strain M/3/R) + III S Vaccine, Heated for 15' at 100°C.*

TABLE XI

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in colonies | | Agglutination H.B. culture |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 4 Controls | 90 | Nil. | All survived. Sacrificed 9 days | All cultures negative | | |
| 6 | 90 | 0.25 | 1 d. 1½ days | R and S | S | Type III |
| | | | 2 " 2 " | " " " | S | " " |
| | | | 3 " " " | " " " | S | " " |
| | | | 4 " " " | " " " | S | " " |
| | | | 5 s. k. 13 " | R only | — | — |
| | | | 6 s. k. " " | — | — | — |

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*Summary:* No. test mice injected, 6.
Died, 4. Reversion to the homologous S type, 4.
Survived, 2. R organisms recovered when sacrificed, 1.

The preceding experiments show that vaccines prepared from Types II S and III S organisms are equally effective in producing reversion whether heated for 15 minutes at 60°C. or for 15 minutes at 100°C. Type I S vaccine, on the other hand, while effective in causing reversion of 1 R forms to the homologous S type when heated for 15 minutes at 60°C. did not possess this property when heated for 15 minutes at 100°C.

Two possible explanations may be advanced for the apparent differences in effect produced by Type I S vaccine heated at 60° and at 100°C. First, that property of the vaccine responsible for reversion might have been destroyed, in the case of Type I vaccine, by heating at 100°C. but not in the case of Type II and Type III vaccines similarly

treated. Second, the failure of Type I vaccine to effect reversion when heated at 100°C. might not have been due to the destruction of any property of the vaccine itself, but rather to the difficulty which had always been encountered in effecting the R→S change with this particular 1 R strain. It has been repeatedly shown that the 2 R and 3 R strains employed in these experiments can be much more readily converted to the S type. However, in view of the entirely similar results recorded by Griffith, and because of the findings to be reported in the subsequent paper, the former explanation is much the more probable.

### *Attempts to Effect the R→S Change by the Injection of Living R Organisms Together with the Heat-Killed Bacteria from Large Amounts of R Cultures*

It was thought that the effect of the vaccines in producing reversion might be due to one of two causes:—First, the injection of such large amounts of heat-killed culture might overwhelm the general resistance of the animal and so allow the R forms to grow in an environment suitable for the development of S types. Second, the vaccines might act locally to protect the R forms from phagocytosis, and so enable them to survive and produce their own S substance. In either case it was thought that the vaccine of an R culture, if injected in sufficiently large amounts together with living R organisms, would similarly allow reversion to take place.

*2 R Culture + 2 R Vaccine of the Same Strain Heated for 15′ at 60°C. (Strain D/39/R).*

TABLE XII

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. cultures |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 2 | 200 | 0.25 | 1 d. 2 days | R only | R only | R |
| | | | 2 s. k. 11 " | — | — | — |
| 4 | 150 | 0.25 | 1 d. ½ days | R only | R only | R |
| | | | 2 " 1 " | " " | R only (2 colonies) | R |
| | | | 3 " 2½ " | " " | R only (few) | R |
| | | | 4 s. k. 11 " | — | — | — |
| 4 | 100 | 0.25 | 1 d. 1 day | R only | R only | R |
| | | | 2 " 1½ " | " " | " " (8 colonies) | R |
| | | | 3 " " " | " " | R only (6 colonies) | R |
| | | | 4 " " " | " " | R only | R |

d; died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*3 R Culture + 3 R Vaccine of the Same Strain Heated for 15′ at 60°C. (Strain M/3/R).*

TABLE XIII

| No. mice | Amount of culture from which heat-killed organisms were obtained | Amt. of living R culture | Result | Kinds of colonies recovered in cultures | | Agglutination H.B. cultures |
|---|---|---|---|---|---|---|
| | | | | P.I. | H.B. | |
| | *cc.* | *cc.* | | | | |
| 2 | 200 | 0.25 | 1 d. 1 day | R only | R only | R |
| | | | 2 s. k. 11 " | — | — | — |
| 4 | 150 | 0.25 | 1 } | — | — | — |
| | | | 2 } s. k. 11 days | — | — | — |
| | | | 3 } | — | — | — |
| | | | 4 } | — | — | — |
| 4 | 100 | 0.25 | 1 d. ½ " | R only | R only | R |
| | | | 2 " 1 " | " " | R only (6 colonies) | R |
| | | | 3 } s. k. 11 " | — | — | — |
| | | | 4 } | — | — | — |

d, died.
s; survived.
k; killed.
P. I. place of injection.
H. B. heart's blood.

*Summary:* (Tables XII and XIII).
No. test mice injected, 20.
Died, 11. Reversion to the homologous S type, 0.
No reversion, only R organisms recovered, 11.
Survived, 9. R organisms recovered when sacrificed, 0.

R vaccines, even when heated for so short a period as 15 minutes at 60°, and in huge doses, representing the bacteria from 200 cc., 150 cc., and 100 cc. of broth culture, completely failed to cause the R cultures to revert to their own type. Similar negative results were obtained when vaccines of S Friedländer bacilli were inoculated together with the R forms of Pneumococcus. Francis (7), in this laboratory, has made similar observations while working with rabbits. He found that S vaccines were effective in causing R forms to revert to the S type;

while R vaccines and vaccines of Staphylococcus failed to produce the change.

### *"In Vitro" Attempts to Effect the R→S Change by Growth of R Forms with S Cultures and with S Vaccines*

(1) In previous work experiments had been done to observe the effect of growing R and S cultures in symbiosis. R and S forms of the same strain were grown together for serial transfers in varying dilutions, as follows:—

| | *cc.* | | | | | | |
|---|---|---|---|---|---|---|---|
| Dilution of S Culture | $10^{-7}$ | $10^{-6}$ | $10^{-5}$ | $10^{-4}$ | $10^{-3}$ | $10^{-2}$ | $10^{-1}$ |
| Dilution of R Culture | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ | $10^{-6}$ | $10^{-7}$ |

0.1 cc. quantities of each of the above R and S dilutions were seeded together in blood broth and daily plates and subcultures made from the mixtures. It was obvious that in such experiments considerable variation in results would be obtained, but generally speaking, the cultures maintained the same relative proportions of R and S colonies for several transfers. Apparently the mere presence and growth of S cells in intimate association with R forms was not sufficient to cause the latter to revert to the S variety.

(2) R organisms were cultured in blood broth to which was added the bacteria from 100 cc. of homologous S culture, heated for 15 minutes at 60°C. Transfers were continued for fifteen subcultures without the appearance of S colonies. At the end of this time the virulence of the R culture was determined by mouse inoculation and found to be unchanged. Such experiments suggest that the R form is not able, under ordinary *"in vitro"* conditions, to utilize an S vaccine, as such, to build up its own polysaccharide.

(3) Moreover, under similar conditions of cultivation, when 10 per cent anti-R serum was added to the media, the change failed to occur; whereas, in the absence of the vaccine, reversion regularly took place after the appropriate number of transfers. Such a result can be explained by assuming that the vaccine, which contains, in addition to its type-specific antigen, the common group-specific protein (or R) antigen (8), absorbed the anti-R antibodies from the immune serum. In the absence of the anti-R antibodies the change failed to occur. This experiment, then, offers further proof of the rôle played by anti-R antibodies in effecting R→ S reversion *"in vitro."*

(4) The possibility that the vaccine might be effective in producing reversion only in the presence of tissues under anaerobic conditions received some consideration. R forms were grown, under vaseline seal, in blood broth to which was added lymph-tissue, muscle tissue and ground up spleen, in addition to large amounts of vaccine. Subcultures were made daily for six days. No S colonies appeared at any time during the process and the cultures remained avirulent.

Thus, under the conditions employed, all attempts to produce the R→S change *in vitro* by the use of vaccines have uniformly failed.

The possibility that the vaccines became "digested" in the subcutaneous tissues of the mice, and that the R forms were able to utilize the "digestion" products to build up their own S substance also received attention. Attempts to reproduce such "digestion" "*in vitro*" will be considered in the subsequent paper.

## DISCUSSION

The conversion of relatively avirulent pneumococci into highly virulent organisms is obviously a matter of considerable biological and epidemiological significance. Recent observations made in this laboratory suggest that R Pneumococci are not infrequently found in the flora of the upper respiratory tract of normal individuals. Such observations indicate that this form of the organism appears not only under artificial conditions of cultivation in the test-tube but may be considered as an evidence of biological adaptation to environment on the part of the bacteria. Moreover, the avirulent R form is potentially capable of again developing into the virulent S type under favorable circumstances. The factors determining such development in the human are difficult of analysis but under experimental conditions certain observations can be made in animals.

Attention is drawn first to the "in vitro" method of producing the R→S change,—the growth of R organisms in anti-R serum. In this connection the existence of anti-R antibodies in the sera of normal individuals, as shown in a previous paper (2), is believed to be a point of considerable significance, and may play a rôle in the R→S reversion process in the human being.

Griffith's observations on the conversion "in vivo" of avirulent R pneumococci into virulent, type-specific, S, organisms have been completely confirmed. In attempting an analysis of the causes responsible for reversion by the technique adopted by him certain points must be considered. He suggests that the mass of culture forms a nidus in which the attenuated pneumococci are protected from the bactericidal action of the tissues. But, as he himself indicates, this can play only a small part in the reversion process. Large amounts of R vaccine, amounts larger than those of the S vaccines

employed, as well as vaccines of other organisms, should also suffice to protect the R forms from phagocytosis and from the bactericidal action of the tissues. Nevertheless, reversion has never been effected under those conditions. Moreover, the finding of living R organisms in the subcutaneous tissues as late as twenty days after injection proves that opportunity to survive is not the only condition necessary to bring about the R→S change in the animal body. Other factors must play a rôle in the reversion process. Is it possible that the S vaccine, disintegrating in the animal tissues, supplies a suitable pabulum from which the living R organisms are able to resynthesize their own specific soluble substance?

A point of considerable difficulty in such a hypothesis is the explanation of the differences in results obtained by the use of Type I S vaccine heated at 60° and at 100°C. It is necessary to assume that Type I S vaccine, when heated at the higher temperature, becomes altered in such a way that it, or its disintegration products, can no longer be utilized by the R forms. But it has been shown (4) that heating at 100° in no way alters the specific carbohydrate fraction of the Type I Pneumococcus. This hypothesis, therefore, is inadequate unless it can be shown that the portion of the vaccine effective in causing reversion in some fraction other than the specific soluble substance as such.

The fact that large amounts of living R cultures by themselves produce the same effect as small amounts of live R cultures when the latter are injected together with S vaccines, suggests that the causes responsible for reversion under these two conditions must be closely related. In this connection it is of importance to point out that all the R strains used in these experiments yielded traces of the specific soluble substance of their original S type. This has been shown in this laboratory by Julianelle (9) in the case of the 1 R strain which offered the greatest resistance to reversion. It is reasonable, then, to assume that large amounts of living R cultures may yield a sufficient amount of S substance, (or the closely related substance necessary for reversion) from which the R forms may resynthesize their own specific polysaccharide. And apparently, once the process is initiated, it is carried on indefinitely, so long as the environment is suitable. However, on this basis, it remains difficult to explain why large amounts

of R vaccines fail to provide the necessary material; while relatively small amounts of living R cultures are able to supply this factor. It may be that the necessary substance is not present in sufficient quantities in the R vaccines; while it is elaborated in an adequate amount by the growth of the living R forms.

The failure of "*in vitro*" attempts to effect reversion by the aid of vaccines deserves some consideration. It was not found possible to induce the R→S change by growing R forms in symbiosis with S organisms, or in media containing large amounts of S vaccines. Likewise, attempts to produce the change by growing R Pneumococci with S vaccines under partial anaerobiosis in the presence of animal tissues were unsuccessful. The failure to effect reversion by these procedures suggests that either the conditions, as provided, were not adequate, or that some factor must be provided by the animal body.

It has been suggested that the S vaccine may possess some "activating" or "co-ferment" property, which, working in conjunction with the synthesizing enzymes of the R form, enables the latter to build up its own S structure. If the effect of the S vaccine is due to such a property it apparently can exercise this function only in the presence of living tissues. Moreover, one is confronted with the difficulty of explaining how such a property becomes inactivated, in the case of Type I S vaccine, by heating at 100°C., and not in the cases of Type II S and Type III S vaccines similarly treated.

Another possibility is that the conditions created in the mouse by the injection of S vaccines may be the determining factors in inducing the R→S change. It has been pointed out previously that all the R cultures used in these experiments yielded traces of specific soluble substance of their original S type. Nevertheless the animals were able to withstand the injection of comparatively large amounts of living R organisms by themselves. The mouse must therefore possess some capacity to overcome infection by organisms producing minimal amounts of S substance. It is possible that the injection of S vaccines may destroy or inhibit this limited ability of the mouse. Under such conditions the R forms may elaborate S substance in greater quantities, and as a consequence develop into S organisms.

In this connection attention is directed to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus,

he reported the following observation. "The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers."

However, in any of the explanations considered, it is impossible to account for the different effect produced by Type I vaccine heated at 60°C and at 100°C. The exact causes responsible for reversion, under these experimental conditions, therefore remain unexplained. Whatever they may be, the fact remains that when R cultures are injected in large amounts by themselves, or in small amounts together with the heat-killed vaccines of S organisms, the characteristics of the R organisms are actually altered. Comparable phenomena may play a rôle of great importance in many infectious processes. A focus of infection may be a point at which relatively harmless organisms assume virulent characteristics; for the subcutaneous injection, in white mice, of large amounts of avirulent pneumococci produces conditions quite analogous to those existing in a focus of infection.

## SUMMARY

R forms of Pneumococcus may be converted into S forms of the homologous Type. In addition to the methods previously reported,—(1) animal passage and (2) growth in anti-R sera,—conversion may be effected by the following procedures as employed by Griffith; (1) The subcutaneous injection, in white mice, of large amounts of living R organisms. (2) The subcutaneous injection, in white mice, of small amounts of living R organisms together with the heat-killed bacteria from large amounts of homologous S cultures. There are "varying degrees of constancy of the R variant"; but by these means it has been possible to effect conversion of all R forms selected. Attempts to cause a further "degradation" of R organisms by continued growth in homologous immune serum have been unsuccessful.

Type II S and III S vaccines are equally effective in producing conversion when heated for 15′ at 60°C., or for 15′ at 100°C. Type I S vaccine, however, while effective in causing conversion when heated for 15′ at 60°C., apparently loses this property when heated for 15′ at 100°C.

R vaccines, and vaccines of other organisms, when injected together with live R cultures, have always failed to produce conversion.

The causes responsible for conversion under these experimental conditions are discussed and the possibility of the occurrence of a similar process under natural conditions in human beings is indicated.

## CONCLUSIONS

1. R forms of Pneumococcus may be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of large amounts of living R organisms.

2. R forms of Pneumococcus may similarly be converted into S forms of the homologous type by the subcutaneous injection, in white mice, of small amounts of living R organisms, together with the heat-killed bacteria from large amounts of S cultures.

3. By these methods Types II S and III S vaccines are equally effective in producing conversion when heated for 15′ at 60°C., or for 15′ at 100°C. Type I S vaccine is effective in producing conversion when heated for 15′ at 60°C., but not when heated for 15′ at 100°C.

4. R vaccines and the vaccines of other organisms are not effective in producing conversion.

5. All "*in vitro*" attempts to produce conversion by the use of vaccines have been unsuccessful.

6. The rôle which the phenomenon of conversion may play in infectious processes is indicated.

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# THE TRANSFORMATION OF PNEUMOCOCCAL TYPES

## II. The Interconvertibility of Type-Specific S Pneumococci

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In the previous communication (1) it was shown that avirulent, non-type-specific, R forms of Pneumococcus could be converted into virulent, type-specific, S forms of the original type by the following procedures as employed by Griffith (2): 1) The subcutaneous injection, in white mice, of large amounts of living R forms: 2) The subcutaneous injection, in white mice of small amounts of living R forms together with the heat-killed bacteria from large quantities of the *homologous* S culture. By these procedures the R forms were invariably converted to S forms of the same specific type as that from which they had been originally derived.

Griffith further reported (2) that R forms of Pneumococcus, derived from S forms of any specific type, could be converted into type-specific organisms of *heterologous* S types by the following procedure:—the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat-killed bacteria from large quantities of *heterologous* S cultures. In other words, he stated that it was possible to transform S Pneumococci of one specific type into other specific types through the intermediate stage of the R form. The present communication is concerned with the question of such transformation of Pneumococcal types.

### *Methods*

The suspensions of heat-killed organisms were prepared in the same manner as described in the preceding paper (1). Similar controls were employed to eliminate the possibility of the existence of viable organisms in the vaccines.[1] In all critical experiments the number of control animals injected was equal to the number of

[1] The term vaccine indicates a suspension of heat-killed pneumococci.

test animals. The bacteria obtained from large quantities of culture, killed by heating, were also injected into animals together with cultures of other live organisms. In addition large amounts of vaccine were injected into animals intoxicated with alcohol. In no instance were viable pneumococci recovered from the controls. Notwithstanding the conclusive nature of the controls employed, even more convincing evidence as to the improbability of the existence of any viable organisms in the heat-killed suspensions will be offered in the subsequent description of the experiments.

The R strains which were used were derived in all instances from typical, type-specific, S pneumococci by growth in homologous immune serum. The possibility of the cultures containing a mixture of R forms, derived from S forms of more than one type, was eliminated in many experiments by the use of single-cell strains. In addition, proof of the nature of the R strains was obtained by converting them to the S form by growth in media containing anti-R serum (3). Under these conditions the R forms invariably reverted to the S form of the same specific type as that from which they were originally derived.

## EXPERIMENTAL

### *Conversion of R Forms of Pneumococcus into S Forms of Heterologous Type by the Subcutaneous Injection, in White Mice, of Small Amounts of Living R Forms, Together with Large Amounts of Heat-Killed S Forms of Heterologous Type*

In the course of early experiments it became apparent that the selection of the R strain played an important rôle in determining the results obtained. The particular 2 R culture (strain D/39/R) which was first chosen could be readily converted to the S form of the original type by all the methods which have been described: 1) animal passage: 2) growth in media containing anti-R serum; 3) the subcutaneous injection, in white mice, of large amounts of living R forms: 4) the subcutaneous injection, in white mice, of small amounts of living R forms together with the heat killed bacteria from large amounts of the *homologous* S culture.

In the first experiment twelve mice were injected with 0.25 cc. of living 2 R culture together with the bacteria from 90 cc. of a Type I S culture, heated for 15′ at 60°C. All the mice died after an interval of 24 to 48 hours and typical Type II S pneumococci were recovered from the heart's blood of each animal. In all cases the R forms were converted to S forms of the same type as that from which they were originally derived.

Another 2 R culture, (strain N/D/39/R), was then obtained by growing a

typical Type II S pneumococcus in its homologous immune serum. After six transfers the culture was plated. An individual colony was then selected and transferred to blood broth. This 2 R culture could likewise be converted into S forms of the original type; but greater difficulty was experienced in bringing about the change than in the case of the 2 R culture previously employed. This second 2 R culture was injected into a series of twelve mice together with aliquot portions of the Type I S vaccine used in the preceding experiment. Quite different results were obtained. Ten of the twelve animals died after an interval of 24 to 48 hours and from the heart's blood of nine of these typical Type I S pneumococci were recovered. Cultures made from the heart's blood of the tenth mouse yielded only R forms. Two mice survived.

TABLE I

*Conversion of R Forms of Pneumococcus into S Forms (1) of the Original Type (2) of the Type of the Vaccine*

| Type and amount of vaccine | Amount of living R culture | Number of mice | Result | Pneumococci recovered by culture | |
|---|---|---|---|---|---|
| | | | | R and S forms | No. mice |
| Bacteria obtained from 90 cc. Type I S culture, heated at 60°C. for 15′. | Nil. | 10 | All survived; sacrificed 7 days. | All cultures negative. | 10 |
| ditto. | 0.25 cc. 2 R (strain D/39/R) | 12 | All died 1–2 days. | Type II S | 12 |
| ditto. | 0.25 cc. 2 R (strain N/D/39/R) | 12 | 10 died 1–2 days.<br>2 survived; sacrificed 7 days. | Type I S<br>R only.<br>All cultures negative. | 9<br>1 |

The results obtained by injecting these two different 2 R strains, together with the same Type I S vaccine, appear in Table I.

*Abstract of Protocol.*—

2 R culture, (strain D/39/R), + I S Vaccine, heated for 15′ at 60°C.
Number of mice injected—12.
Number of mice died—12.
Reversion to S forms of the original type—12.
Reversion to S forms of the same type as the vaccine—0.

2 R culture, (strain N/D/39/R), + I S Vaccine, heated for 15′ at 60°C.
Number of mice injected—12.
Number of mice died—10.

Reversion to S forms of the original type—0.
Reversion to S forms of the same type as the vaccine—9.
No reversion, only R organisms recovered—1.
Number of mice survived—2.

The Type I S organisms which were recovered from the heart's blood of the second series of mice possessed all the attributes of typical Type I S pneumococci. The cultures agglutinated specifically in Type I serum; they elaborated the specific soluble substance characteristic of Type I pneumococci, and were highly virulent for white mice. Subcultures made through twenty transfers retained the same properties and the organisms showed no tendency to revert to their original S type.

Since the same Type I S vaccine was used in the two experiments the variation in results obtained must have been referable to a difference in the R cultures employed. This difference was reflected in the greater difficulty experienced in causing the second 2 R culture to revert to the S form of its original type. Apparently in order that an R culture may revert to the S form of a heterologous type it must first be reduced to a definite stage in the "degradation" process.

This experiment also offers further proof of the absence of any viable organisms in the vaccine. It is highly improbable that the Type I S organisms recovered from the second series of mice could have developed from surviving forms in the vaccine; for no such forms were recovered from either the control mice or from the first series of mice which received the same vaccine.

An interesting result was obtained when still another 2 R culture was injected into mice along with Type I S vaccine. This third 2 R culture was obtained in the usual way by growing a typical Type II S pneumococcus in its homologous immune serum. When grown in anti-R serum, or injected subcutaneously in white mice in large amounts by itself, this R culture invariably reverted to the S form of the same type as that from which it was originally derived. Eleven mice were injected subcutaneously with 0.25 cc. of living R forms together with the bacteria from 100 cc. of Type I S culture heated for 15′ at 60°C. Eight out of the twelve animals died after a period of one to two days, and Type I organisms were recovered from the heart's blood. In certain respects, however, the organisms recovered were not typical S forms. Although the cultures agglutinated specifically in Type I serum and produced the specific soluble substance characteristic of Type I pneumococcus, they did not possess maximal virulence for white mice nor

did they produce typical Smooth colonies. As a rule the cultures proved fatal to white mice in dilutions of $10^{-5}$ cc., occasionally in dilutions of $10^{-6}$ cc., but never in dilutions of $10^{-7}$ cc. The colonies were atypical in appearance, having neither the glossy, shiny surface of Smooth colonies nor the finely granular, ground-glass appearance of Rough colonies. For the most part the colonies were irregular in outline with nibbled margins. The outer zone was usually slightly rough; toward the center the majority presented a smooth wavy appearance; and in the middle of the colony there was frequently a crater-like depression. Other colonies more closely resembled typical R colonies and still others were suggestive of those of the S variety. That such colonies were not composed of a mixture of Rough and Smooth organisms was repeatedly proven by selecting individual colonies, subculturing, and plating for several transfers. Even when passed through mice colonies with the same characteristics were recovered from the heart's blood. These forms most probably represented incompletely developed S organisms partially "stabilized" at this phase of the reversion process. Such cultures could be converted into typical S forms by the subcutaneous injection in white mice of large amounts of the cultures alone.

In the course of the same experiment a 3 R culture, obtained by growing a typical Type III S pneumococcus in Type III serum, was injected into a series of eleven mice, together with aliquot portions of the same Type I S vaccine. Seven of the eleven animals succumbed and typical Type I S organisms were recovered from the heart's blood in each instance. All the colonies obtained from this series of animals were of the typical Smooth variety, and no "intermediate" forms were observed. All the cultures possessed maximal virulence for white mice.

Since the heat-killed suspension used in both series was the same the conclusion must be drawn that the atypical intermediate colonies recovered from the first series of mice developed from the 2 R culture and not from the vaccine. This observation offers still further proof of the absence of viable forms in the heat-killed suspensions.

In certain other experiments the injection of an R culture together with a heterologous S vaccine resulted in the recovery of a mixture of S forms from the animals. Such mixtures were composed exclusively of S organisms of the same type as the vaccine and the type from which the R forms had been originally derived. In all such cases S organisms were not obtained from any of the control mice. It must therefore be concluded that, in these instances, conditions were equally suitable for reversion of the R forms to the S forms of the original type, or to the type of the vaccine.

When a plate is composed of a mixture of colonies of various S types the Type III colonies are usually readily identified by their large size and clear, watery appearance. It is also possible, as a rule, to identify colonies composed of Type I S organisms. When examined against a dark background through a plate culture microscope Type I colonies are usually denser, more opaque and whiter than Type II colonies. Colonies composed of Type II and Group IV organisms, on the other

hand, are paler, more transparent and "watery." They also appear to undergo autolysis more readily than Type I colonies and frequently present a 'ring' or 'life-saver' appearance.

Experiments were next undertaken to determine whether one and the same R culture could be successively transformed into the S form of each of the specific types of pneumococcus.

A 2 R culture was obtained by growing a typical Type II pneumococcus in its homologous immune serum. After four transfers the culture was plated. A single R colony was selected and subcultured in blood broth. A single-cell strain was then obtained from this culture by the method of Avery and Leland (4). Four mice were injected with 0.25 cc. of the single-cell culture together with the bacteria from 100 cc. of a Type III S culture heated for 30′ at 60°C. Three of the animals succumbed, and typical Type III S pneumococci, possessing all the characteristics of that type, were obtained from the heart's blood of each. One of these III S cultures was then converted into the R form by growth in Type III serum. After five transfers the culture was plated. A single R colony was selected and subcultured in blood broth. The resultant growth was plated and again a single colony was selected and subcultured. This process was repeated four times and the final culture was injected into four mice together with a Type I S vaccine heated for 30′ at 60°C. All four mice died and typical Type I S organisms were recovered from the heart's blood in each instance. One of these typical Type I S cultures was again converted to the R form in the same manner as previously described. The resulting R culture was injected into four mice together with a vaccine prepared from a Group IV S culture. Two of the mice died yielding S organisms in the heart's blood. Cultures from the heart's blood of these two animals did not agglutinate specifically in Types I, II or III sera but were highly virulent for mice. Specific anti-serum was not available to test the agglutination of the Group IV S strains, but in view of the preceding results it was highly probable that the cultures were of the same variety as the Group IV vaccine.

During the various stages of this experiment whenever an R culture was obtained it was grown in media containing 10 per cent anti-R serum. In all cases, after a variable number of transfers, the R form was converted to the S form of that type from which it had last been derived. This observation lends support to the contention that in no instance was a mixture of R forms, derived from S forms of more than one type, present in the culture.

In summary, a typical Type II S pneumococcus was successively transformed, through the intermediate stage of the R form, into a Type III S pneumococcus, a Type I S pneumococcus, and a Group

TABLE II

*The Effect of the Temperature at Which a Type I S Vaccine is Heated upon Its Efficacy in Inducing Transformation of Type*

| Type and amount of vaccine | Temp. at which vaccine was heated for 15′ | Amount of living R culture | Number of mice | Result | Pneumococci recovered by culture | |
|---|---|---|---|---|---|---|
| | | | | | R and S forms | N mice |
| | C. | | | | | |
| Bacteria obtained from 100 cc. Type I S culture | 60° | Nil | 4 | All survived; sacrificed 8–10 days | All cultures negative | 4 |
| ditto | 60° | 0.25 cc. 2R | 5 | All died 1–2 days | Type I S | 5 |
| ditto | 65° | Nil | 2 | Both survived; sacrificed 8–10 days | All cultures negative | 2 |
| ditto | 65° | 0.25 cc. 2R | 5 | All died 1–2 days | Type I S | 5 |
| ditto | 70° | Nil | 2 | Both survived; sacrificed 8–10 days | All cultures negative | 2 |
| ditto | 70° | 0.25 cc. 2R | 5 | All died 1–2 days | Type I S | 5 |
| ditto | 75° | Nil | 2 | Both survived; sacrificed 8–10 days | All cultures negative | 2 |
| ditto | 75° | 0.25 cc. 2R | 5 | All died 1–2 days | Type I S | 5 |
| ditto | 80° | Nil | 2 | Both survived; sacrificed 8–10 days | All cultures negative | 2 |
| ditto | 80° | 0.25 cc. 2R | 5 | All died 1–2 days | Type I S | 5 |
| ditto | 100° | Nil | 2 | Both survived; sacrificed 8–10 days | All cultures negative | 2 |
| ditto | 100° | 0.25 cc. 2R | 5 | Three died 1–2 days | R only | 3 |
| | | | | Two survived; sacrificed 8–10 days | All cultures negative | 2 |

IV S pneumococcus. At any stage of the cycle the R form could be converted to the S form of that type from which it had last been derived by growth in anti-R serum.

### *The Effect of the Temperature at Which an S Vaccine Is Heated upon Its Efficacy in Causing an R Culture, Derived from a Heterologous S Type[2] to Revert to the Type of the Vaccine*

Griffith reported (2) that S vaccines, when heated at temperatures higher than 70°C., were rarely effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To verify this finding the following experiment was devised.

The bacteria obtained by centrifuging 4600 cc. of a Type I S culture were suspended in 23 cc. of plain broth. This suspension was divided into four equal portions of 3.5 cc. each, and two portions of 4.5 cc. each. (The larger portions were heated at the lower temperatures and a greater quantity of suspension was required for additional controls.) Each of these six portions of the same vaccine was heated for fifteen minutes at varying temperatures between 60°C. and 100°C., as outlined in the accompanying table. After heating, a volume of 2 cc. from each of the larger samples, and 1 cc. from each of the smaller samples was reserved for injection into control mice. To the remaining amounts of each portion of vaccine 1.25 cc. of a blood broth culture of a 2 R pneumococcus was then added. The quantities were so arranged that all mice, including the controls, received the heat-killed bacteria from 100 cc. of culture. In addition, each test animal received 0.25 cc. of living 2 R culture.

The results of the experiment appear in Table II.

*Abstract of Protocol.*—Type I S vaccine, heated for a period of 15′ at temperatures between 60°C. and 80°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R culture to revert to a Type I S pneumococcus. Type I S vaccine heated for 15′ at 100°C. does not possess this property.

Attention should be called to certain features in this experiment. In the first place an unusually large number of transformations was obtained. Other experiments were not so uniformly successful. In another experiment a Type I S vaccine, heated for 15′ at 70°C. caused a 2 R culture to revert to the type of the vaccine but did not do so when heated for 15′ at 80°C. Again, in the experiment recorded above, the Type I S vaccine heated at 100°C. apparently had no effect on the 2 R culture. It did not even cause the R culture to revert to its original S type. In other experiments, however, a Type I S vaccine, heated for 15′ at temperatures of 90° and 100°C., caused both 2 R and 3 R cultures to revert to their original S types. The results of such an experiment are reported in Table III.

[2] More correctly this should read "derived from the S form of a heterologous type." For the sake of brevity in this and succeeding instances the above expression has been adopted.

*Abstract of Protocol.*—In the experiment detailed in Table III Type I S vaccine, heated for 15′ at 70°C., was effective in causing a 2 R culture to revert to a Type I S pneumococcus. When heated for the same period at a higher temperature than 70°C. Type I S vaccine was not effective in causing a 2 R culture to revert to the type of the vaccine. However, in several mice which received the Type I S vaccine heated at 80°, 90° and 100°C. the 2 R forms reverted to their original S type.

TABLE III

*The Effect of Temperature at Which a Type I S Vaccine Is Heated upon Its Efficacy in Inducing Transformation of Type. (Second Experiment)*

| Type and amount of vaccine | Temp. at which vaccine was heated for 15′ | Amount of living R culture | Number of mice | Result | Pneumococci recovered by culture | |
|---|---|---|---|---|---|---|
| | | | | | R and S forms | No. mice |
| | C. | | | | | |
| Bacteria obtained from 80 cc. Type I S culture. | 60° | Nil | 4 | All survived; sacrificed 7 days | All cultures negative | 4 |
| ditto | 60° | 0.25 cc. 2 R | 4 | All died 1–2 days | Type I S | 3 |
| | | | | | R only | 1 |
| ditto | 70° | Nil | 2 | Both survived; sacrificed 7 days | All cultures negative | 2 |
| ditto | 70° | 0.25 cc. 2 R | 4 | All died 1–2 days | Type I S | 3 |
| | | | | | R only | 1 |
| ditto | 80° | Nil | 2 | Both survived; sacrificed 7 days | All cultures negative | 2 |
| ditto | 80° | 0.25 cc. 2 R | 4 | 3 died 1–2 days; 1 survived | Type II S | 2 |
| | | | | | R only | 1 |
| ditto | 90° | Nil | 2 | Both survived; sacrificed 7 days | All cultures negative | 2 |
| ditto | 90° | 0.25 cc. 2 R | 4 | All died 1–2 days | Type II S | 2 |
| | | | | | R only | 2 |
| ditto | 100° | Nil | 2 | Both survived; sacrificed 7 days | All cultures negative | 2 |
| ditto | 100° | 0.25 cc. 2 R | 4 | 3 died 1–2 days; 1 survived | Type II S | 2 |
| | | | | | R only | 1 |

Similar results were obtained when a 3 R culture was injected into mice together with a Type I S vaccine heated at various temperatures. From the majority of the animals which received the Type I S vaccine heated at temperatures up to 80°C. S organisms of the same type as that of the vaccine were obtained. From the animals which received the Type I S vaccine heated at higher temperatures than

80°C. no Type I S organisms were recovered. Several of these mice, however, did yield typical Type III S pneumococci.

These findings may be summarized as follows:—Type I S vaccine, heated for 15′ at various temperatures between 60° and 80°C. is effective in causing an R culture derived from a heterologous S type

TABLE IV

*The Effect of the Temperature at Which Types II S and III S Vaccines Are Heated upon Their Efficacy in Inducing Transformation of Type*

| Type and amount of vaccine | Temp. at which vaccine was heated for 15′ | Amount of living R culture | Number of mice | Result | Pneumococci recovered by culture | |
|---|---|---|---|---|---|---|
| | | | | | R and S forms | No. mice |
| | C. | | | | | |
| Bacteria obtained from 90 cc. Type II S culture. | 60° | 0.25 cc. 1 R | 6 | 3 died 3 survived | Type II S | 3 |
| ditto | 60° | 0.25 cc. 3 R | 8 | All died | Type II S | 8 |
| ditto | 100° | 0.25 cc. 1 R | 6 | All survived | | |
| ditto | 100° | 0.25 cc. 3 R | 6 | 5 died 1 survived | Type III S | 5 |
| Bacteria obtained from 90 cc. Type III S culture. | 60° | 0.25 cc. 1 R | 6 | 4 died 2 survived | Type III S | 4 |
| ditto | 60° | 0.25 cc. 2 R | 6 | 4 died 2 survived | Type III S | 4 |
| ditto | 100° | 0.25 cc. 1 R | 6 | All survived | | |
| ditto | 100° | 0.25 cc. 2 R | 6 | 3 died 3 survived | Type II S | 3 |

to revert to the type of the vaccine. When heated at temperatures higher than 80°C. Type I S vaccine does not cause an R culture derived from a heterologous S type to revert to the type of the vaccine; but frequently causes the R culture to revert to its original S type.

Experiments were then undertaken to determine whether vaccines prepared from S organisms other than Type I were subject to the same thermal differentiation as a Type I S vaccine.

(In the previous paper it was shown that vaccines prepared from cultures of Types II S and III S pneumococcus, whether heated at 60°C. or 100°C., were equally effective in causing R organisms, derived from the same S type as that of the vaccine, to revert to their original S type. It was also shown that vaccines prepared by heating cultures of Type I S pneumococcus at 60°C. were effective in causing a 1 R culture to revert to its original S type. However, when a Type I S vaccine was heated at 100°C. this property was destroyed and reversion failed to occur.)

Vaccines were prepared by heating a culture of Type II S pneumococcus at temperatures of 60°C. and 100°C. Equal portions of each lot of vaccine were injected into two series of mice together with 1 R and 3 R cultures, respectively. Similarly, vaccines were prepared by heating a culture of Type III S Pneumococcus at 60°C. and at 100°C. Two series of mice were injected with these vaccines together with 1 R and 2 R cultures, respectively.

The results of these experiments appear in Table IV.

From the foregoing results and from those obtained in previous experiments the following conclusions may be drawn:—

(1) Vaccines prepared by heating cultures of each of the three S types of pneumococcus at 60°C. are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(2) Vaccines prepared by heating cultures of each of the three S types at 100°C. are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(3) Vaccines prepared by heating cultures of each of the three S types at 100°C. are frequently effective in causing 2 R and 3 R cultures to revert to the same specific type from which they were originally derived.

(4) Vaccines prepared by heating cultures of any S type, including Type I, at 100°C. are not effective in causing a 1 R culture to revert to its original S type.

### *The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Causing an R Culture, Derived from a Heterologous S Type, to Revert to the Type of the Vaccine*

Griffith reported that vaccines heated for long periods, even at temperatures as low as 60°C., were less effective than vaccines heated for short periods in causing R forms, derived from heterologous S types, to revert to the type of the vaccine. To test the effect of heating an S vaccine for varying periods the following experiment was arranged.

The bacteria obtained by centrifuging 4000 cc. of a Type I S culture were taken up in 20 cc. of plain broth and heated for 15′ at 60°C. A quantity of 8 cc. of suspension was withdrawn and used in the first part of the experiment. The remaining 12 cc. of suspension were heated for a further period of 15′ at the same temperature. Three cc. were then withdrawn and a like amount at half-hour intervals thereafter on three occasions. Five cc. of the first 8 cc. sample withdrawn were used for control purposes, a volume of 0.5 cc. being injected subcutaneously into each of ten mice. To each of the five 3 cc. samples 1.5 cc. of a living 2 R

TABLE V

*The Effect of the Duration of Heating upon the Efficacy of a Type I S Vaccine in Inducing Transformation of Type*

| Type and amount of vaccine | Length of time during which vaccine was heated at 60°C. | Amount of living R culture | Number of mice | Result | Pneumococci recovered by culture | |
|---|---|---|---|---|---|---|
| | | | | | R and S forms | No. mice |
| Bacteria obtained from 100 cc. Type I S culture. | 15′ | Nil | 10 | All survived; sacrificed at intervals up to 17 days | All cultures negative | 10 |
| ditto | 15′ | 0.25 cc. 2 R | 6 | 5 died<br>1 survived | Type I S | 5 |
| ditto | 30′ | 0.25 cc. 2 R | 6 | 4 died<br>2 survived | Type I S | 4 |
| ditto | 1 hour | 0.25 cc. 2 R | 6 | 5 died<br>1 survived | Type I S | 5 |
| ditto | 1½ hours | 0.25 cc. 2 R | 6 | 4 died<br>2 survived | Type I S | 4 |
| ditto | 2 hours | 0.25 cc. 2 R | 6 | 5 died<br>1 survived | Type I S | 5 |

culture was added. Six test mice were inoculated in each part of the experiment with the heat-killed bacteria from 100 cc. of Type I S culture, together with 0.25 cc. of living 2 R culture.

The details of the experiment are given in Table V.

*Abstract of Protocol.*—Type I S vaccine, heated for as long a period as two hours at 60°C., when injected subcutaneously in white mice together with a living 2 R culture, apparently possesses the ability to cause the 2 R forms to revert to Type I S organisms. A Type I S vaccine heated for 2 hours at 60°C. is just as effective in producing reversion to the type of the vaccine as that heated for 15′ at 60°C.

Mention should again be made of the fact that this also was an unusually successful lot of vaccine. In other experiments a smaller number of positive results was obtained and there appeared to be a slight falling off in the effectiveness of the vaccine when heated for prolonged periods at 60°C.

### *The Amount of Heat Killed S Organisms Necessary to Cause R Forms Derived from a Heterologous S Type, to Revert to the Type of the Vaccine*

The minimal amount of vaccine capable of inducing transformation of type was then ascertained. An experiment was devised in which

TABLE VI

*The Amount of Heat-Killed Suspension Necessary to Induce Transformation of Type*

| Amount of culture from which Type I S vaccine was prepared | Amount of living R culture | Number of mice | Result | Pneumococci recovered on culture | |
|---|---|---|---|---|---|
| | | | | R and S forms | No. mice |
| 100 cc. | 0.25 cc. 2 R | 4 | 4 died | Type I S | 4 |
| 50 cc. | 0.25 cc. 2 R | 4 | 3 died<br>1 survived | Type I S | 3 |
| 25 cc. | 0.25 cc. 2 R | 4 | 2 died<br>2 survived | Type I S | 2 |
| 10 cc. | 0.25 cc. 2 R | 4 | All survived | — | |
| 100 cc. | Nil | 4 | All survived; sacrificed 7 days. | All cultures negative. | 4 |

four series of mice received a living 2 R culture together with varying amounts of Type I S vaccine. In addition to the living R culture the first series of animals received the heat-killed bacteria from 100 cc. of S culture; the second from 50 cc.; the third from 25 cc. and the fourth from 10 cc. Four control mice received only the heat-killed bacteria from 100 cc. of S culture.

The results of this experiment appear in Table VI.

*Abstract of Protocol.*—From the heart's blood of all 4 mice which received the living 2 R culture together with the heat-killed bacteria from 100 cc. of Type I S culture, S organisms of the same type as the vaccine were obtained. Similarly

Type I S organisms were recovered from 3 out of 4 mice which received the heat-killed bacteria from 50 cc. and from 2 out of 4 which received the bacteria from 25 cc. From those which received the bacteria from 10 cc. no Type I S organisms were obtained. The control mice which were injected with the heat-killed bacteria from 100 cc. of Type I culture all survived. They were sacrificed at the end of 7 days and cultures from the blood and viscera were negative.

In many other experiments it has constantly been found that large amounts of vaccine are necessary to effect transformation of type by this procedure.

### *The Effect of Autolysis on the Efficacy of an S Vaccine in Causing R Forms, Derived from Heterologous S Types, to Revert to the Type of the Vaccine*

Early in the work it was found that many lots of vaccine were relatively ineffective in causing R forms to revert to the Type of the vaccine. In searching for an explanation of these failures it was noticed that many mice in such unsuccessful experiments developed purpura to a marked degree. Julianelle and Reimann (5) have shown that the purpura-producing fraction of pneumococcus is released only during autolysis and is not present in heat-killed cultures in which autolysis has not taken place. This fact suggested a possible explanation for the ineffectiveness of certain lots of vaccine. It seemed possible that the cultures might have undergone partial autolysis before being heat-killed and, as a consequence, the vaccines made from such cultures were no longer effective in producing reversion. To test this hypothesis the following experiment was done:—

Cultures of Type I S and Type II S organisms were centrifuged and the deposit divided into two equal portions. One-half of the deposit from each culture was immediately heated at 60°C. for 15′: the other half was allowed to autolyze at 37°C. for 48 hours. At the end of this period the autolysate was subjected to a temperature of 60°C. for 15′. The two preparations were injected into a series of mice together with cultures of heterologous R organisms as detailed in Table VII.

*Abstract of Protocol.*—Autolysates of S cultures, when injected subcutaneously in white mice together with living R cultures, are not effective in causing R forms to revert, either to their original S type, or to the S type from which the autolysate was prepared. (Heat-killed suspensions of S organisms, however, kept in the ice-box for periods up to three weeks, have been found to be effective in causing R forms derived from heterologous S types, to revert to the type of the vaccine.)

This experiment offers evidence that it is not the specific soluble substance as such that is responsible for transformation of type in these procedures. The specific soluble substance of pneumococcus is not altered during autolysis and is present both in the autolysate and in the heat-killed suspension (6).

TABLE VII

*The Effect of Autolysis on the Efficacy of an S Vaccine in Inducing Transformation of Type*

| Type and amount of vaccine | Type and amount of autolysate | Amount of living R culture | Number of mice | Result | Pneumococci recovered on culture | |
|---|---|---|---|---|---|---|
| | | | | | R and S forms | No. mice |
| Bacteria obtained from 80 cc. Type I S culture heated for 15′ at 60°C. | Nil | 0.25 cc. 2 R | 4 | 4 died | Type I S | 4 |
| ditto | Nil | 0.25 cc. 3 R | 4 | 3 died<br>1 survived | Type I S | 3 |
| Nil | Bacteria obtained from 80 cc. Type I S culture allowed to autolyze for 48 hrs. at 37°C. | 0.25 cc. 2 R | 4 | All survived; sacrificed 7 days. | All cultures negative | 4 |
| Nil | ditto | 0.25 cc. 3 R | 4 | All survived; sacrificed 7 days. | All cultures negative | 4 |

*The Effect of Injecting S Cultures Killed by Other Agents than Heat Together with R Cultures Derived from Heterologous S Types*

In the experiments reported up to this point the S cultures were invariably killed by heat.

In order to determine whether or not S organisms killed by other agents than heat possessed similar properties, cultures of S pneumococci were killed with the following substances:—formalin, iodine,

chloroform, tricresol, alcohol and acetone. None of the reagents selected destroy the specific soluble substance of pneumococcus (7). The smallest possible concentrations were employed to eliminate the toxic effect of the reagents themselves.

Six series of five mice each were injected with a living 2 R culture together with the bacteria from 100 cc. of Type I S culture which had been killed by adding minimal amounts of each of the above mentioned bactericidal substances. Reversion to the type of the vaccine did not occur in a single instance. At the same time five animals were injected with the bacteria from 100 cc. of the same Type I S culture, heated for 15′ at 60°C. together with the same living 2 R culture. Type I S organisms were recovered from the heart's blood in each instance. Four control mice which received only the heat-killed vaccine survived.

The failure to induce reversion by the use of suspensions of S organisms killed by other agents than heat suggests one of two possibilities; —either that portion of the vaccine responsible for reversion may have been destroyed by the bactericidal substances; or, the toxic effect of the reagents may have created unfavorable conditions in the tissues of the animals.

### *Results Obtained by the Intraperitoneal Injection of Small Amounts of Living R Forms Together with the Heat-Killed Bacteria from Large Amounts of Heterologous S Cultures*

In all the preceding experiments living R forms and S vaccines were injected *subcutaneously* in white mice Experiments were undertaken to determine whether similar transformations could be effected by the *intraperitoneal* injection of living R forms together with vaccines of S cultures. It was hoped that it would be possible during this procedure to follow the transformation process by withdrawing and examining portions of the peritoneal contents from time to time. The results were not uniformly satisfactory. In one experiment eight mice were injected intraperitoneally with a living 2 R culture together with a Type III S vaccine. Four of the animals died and Type III S organisms were recovered from the heart's blood. However, these organisms were only slightly agglutinable in Type III serum and the colonies were not of the large, typical Type III S variety. Typical Type III organisms were obtained by passing these cultures through second

mice. The first cultures were apparently composed of incompletely developed Type III S forms.

In another experiment varying amounts of a living 2 R culture were injected intraperitoneally together with large quantities of a Type I S vaccine. In no case did the R form revert to the type of the vaccine. It was therefore concluded that, while reversion to the type of the vaccine could be effected intraperitoneally, the subcutaneous route was the method of choice.

### *Attempts to Determine the Minimal Time Required to Effect Reversion of R Forms into Organisms of Heterologous S Type*

In different experiments there was a great variation in the interval required to effect reversion. The shortest time in which an animal succumbed, yielding S organisms of the same type as that of the vaccine, was eighteen hours; the longest nine days. In some experiments virulent S organisms were recovered at the site of injection from apparently healthy animals which were sacrificed at the end of seven days. The finding of virulent bacteria in local abscesses in otherwise healthy animals after such an interval suggested that the mice had acquired considerable general immunity before the R forms had had the opportunity to develop into the S form. In the most successful experiments the usual time at which the animals succumbed was from one and one-half to two days after injection. However, if, in this period, S forms had developed in sufficient numbers to overwhelm the animal the actual time necessary for the reversion process to occur at the site of injection was probably much shorter. An attempt was made to determine the minimal time required in the following way.

A series of mice was injected subcutaneously with an S vaccine. Thereafter, at intervals of two, four, eight, twelve, and twenty-four hours living R forms were introduced into the same animals at the same location. Reversion to the type of the vaccine occurred in a moderate number of animals which received the living R forms as late as eight hours after the injection of the vaccine. Reversion did not occur in any animal which received the living culture after an interval longer than eight hours. However, only a small number of animals was employed in this experiment and for this reason the results cannot be considered conclusive. The procedure of introducing the living R forms simultaneously with the vaccines uniformly gave a higher proportion of positive results.

*Attempts to Effect Reversion by the Injection of Living R Forms and S Vaccines in Different Locations in the Same Animals*

Various ways in which S vaccines might act in causing reversion of R forms in the animal body were considered. One of the possibilities was that the general conditions created in the animal by the injection of such large amounts of vaccine might be suitable for the development of organisms of the S variety. If the reversion process depended upon the existence of such general conditions it might be possible to produce a comparable state by the injection of S vaccines and living R forms into different locations in the same animals. Six mice were injected with a Type I S vaccine in one inguinal region. Simultaneously a living 2 R culture was injected into the opposite inguinal region. One of the six mice died in two days and organisms of the same type as the vaccine were recovered from the heart's blood. The other five mice survived. However, such experiments are not conclusive for it is always possible that no matter where the living R forms are injected some organisms may find their way to the site of the vaccine. Because of this difficulty no further experiments were attempted along these lines.

*Attempts to Effect Reversion by the Injection of Living R Forms of Pneumococcus Together with S Vaccines of Friedländer's Bacillus*

It has been demonstrated in this laboratory that Type II pneumococcus and Type B Friedländer's bacillus elaborate specific soluble substances which are chemically and immunologically similar, although not identical (8). Experiments were therefore undertaken to determine whether it was possible to convert R pneumococci, derived from other S types than Type II, into Type II S organisms, by the use of a Friedländer Type B vaccine. R forms of pneumococci, derived from both Type I S and Type III S organisms, were injected into a series of mice together with a Type B Friedländer vaccine. The experiments were accompanied by certain difficulties, for it was found that the primary toxicity of S Friedländer vaccine was considerable.

In the first experiment a series of four mice was injected with the bacteria from 80 cc. of Type B Friedländer culture along with a living culture of 3 R pneumococcus. A similar number of animals was injected with the same Friedländer

vaccine and a culture of 1 R pneumococci. All the animals died after an interval of less than twenty-four hours but only R forms of pneumococci were recovered from the heart's blood.

The converse of the above experiment was then done.

R forms of Friedländer bacilli were injected into mice along with the heat-killed bacteria from a Type II S pneumococcus culture. R forms derived from each of the three specific types A, B, C, and from a strain of the heterogeneous group X were employed. Four mice were injected in each experiment. Two animals died after a period of twelve hours; the remainder survived and were sacrificed after a period of six days. Cultures made from the site of injection and from the heart's blood did not yield S Friedländer bacilli in a single instance. R forms were found at the site of injection in a large proportion of cases. Cultures of the recovered R forms were passed through a second series of mice and the virulence of the organisms was found to remain unchanged. Unfortunately many of the mice in this experiment developed ulcers at the place of injection. It is possible that this fact may have had some effect in determining the results obtained. It is also possible that the particular R strains of Friedländer's bacillus which were selected were not suitable for reversion.

### *Attempts to Convert R Forms of Pneumococci into Organisms of the Heterologous S Type by in Vitro Methods*

The *in vitro* methods which were employed in attempts to convert R forms into organisms of the homologous S type, by the use of vaccines, have been described in a preceding paper (1). All the procedures adopted gave negative results. Similar attempts were made to convert R forms into S organisms of heterologous types. All such experiments were unsuccessful. It must therefore be concluded that, either the *in vitro* conditions, as provided, were inadequate, or that living tissues are essential for the reversion process. An attempt to partially reproduce *in vivo* conditions in the test tube was made in the following way.

Large quantities of a Type I S vaccine were injected intraperitoneally into five mice. The animals were sacrificed at intervals of 2, 4, 8, 12 and 24 hours and the peritoneal contents washed out with plain broth. The washings were transferred to test tubes and seeded with a 2 R culture. Plates made from the resulting growth, however, yielded only R colonies and the cultures remained avirulent for white mice.

The negative results of this experiment suggest that living tissues may play a part in the reversion process. However, it should be pointed out that in previous experiments the intraperitoneal route was not found as suitable as the subcutaneous route. The possibility remains that if the conditions obtaining in the subcutaneous tissues of the mouse could be reproduced in the test tube transformation of type might be effected *in vitro*.

### *Attempts to Convert S Pneumococci of One Specific Type Directly into S Organisms of Another Specific Type*

In all the experiments described in which pneumococci have been converted from one specific S type into other specific S types the transformation has been effected through the intermediate stage of the R form. Inasmuch as the phenomenon of transformation of type had never been observed in type-specific S cultures under artificial cultivation it seemed most unlikely that S organisms could be transformed directly from one specific type to another specific type. An attempt to effect such a direct transformation of type was made in the following manner. Mice were injected subcutaneously with the smallest possible dilutions of living S cultures together with the heat-killed bacteria from large amounts of heterologous S cultures. For example a Type II S culture, in dilutions of $10^{-7}$, $10^{-8}$, and $10^{-9}$ cc. was injected into a series of mice together with a Type I S vaccine. In all cases the animals succumbed and only S organisms of the same type as those introduced in the living cultures were recovered from the heart's blood. Direct transformation from one type to another did not occur in a single instance. These experiments also prove that large quantities of vaccine have no inhibitory effect on any viable forms introduced with the vaccine. On the contrary the animals that received both the living culture and the vaccine succumbed in a much shorter period of time than those which received only the dilutions of living culture.

## DISCUSSION

The transformation of pneumococci from one specific type into other specific types is a phenomenon of wide bacteriological and epidemiological significance. It has not been conclusively demonstrated

that transformation of type actually occurs under natural conditions. Griffith (2) has presented certain evidence which indicates the possibility of such an occurrence during disease processes, but further work is required to establish definitely the validity of these observations. In any case the demonstration that transformation of type may be effected experimentally shows that the various types of pneumococcus are closely related biologically. Indeed it is possible to think that these various types may represent attempts on the part of the organism to adapt itself to varying environmental conditions.

It is important to note that it was found impossible to transform S organisms directly from one specific type into other specific types. Change of type was invariably brought about through the intermediate stage of the R form. The R form of the organism is most readily produced by growing S organisms in their homoglous immune serum. It may also be produced by subjecting S pneumococci to unfavorable environmental conditions,—such as, growth in poor media, growth at temperatures between 40° and 42°C., and growth in media containing small traces of bile. The R form, therefore, probably results from attempts of S bacteria to adapt themselves to unfavorable environmental conditions. Once reduced to the R state the organisms potentially have the capacity to develop the S structure of any of the various specific S types. They most readily assume the characteristics of that S type from which they were last derived; but under the influence of certain conditions they may also develop the S structure of other specific types.

What conditions determine the development of S characteristics? The change may be induced experimentally by subcutaneously injecting, in white mice, large amounts of an S vaccine together with living R forms. The type of S structure which the R forms assume under these conditions is apparently dictated by two circumstances; (1) The degree of "degradation" to which the R forms have been subjected; (2) The degree of heat to which the S vaccine has been exposed. If the R forms have been reduced to a definite state in the "degradation" process they assume the characteristics of the same S type as the vaccine. If the R forms have been only partially "degraded" they assume the characteristics of that S type from which they were originally derived. Similarly, if the vaccine is heated at a tempera-

ture between 60° and 80°C. the R forms revert to the type of the vaccine: if the vaccine is heated at a temperature higher than 80°C. the R forms revert to the S type from which they were originally derived.

What are the causes responsible for transformation of type as induced by this procedure? In the previous paper various possibilities were considered to explain the way in which S vaccines might act in causing R forms, derived from the same S type as the vaccine, to revert to their original S type. It was pointed out that the precise factor responsible for reversion, as brought about by this procedure, was not understood. If the causes determining reversion of R forms to their original S type are not understood it is even more difficult to interpret the conditions under which R forms assume the characteristics of S organisms of the same type as the vaccine. That property of the vaccine responsible for reversion does not exactly correspond with any known substance or property of S organisms. It cannot be the S substance itself, for it has been shown that the carbohydrate fraction of pneumococcus is not altered by heating at 100°C. (9), and its specificity is not destroyed during autolysis. Moreover, the efficacy of an S vaccine in inducing transformation of type does not parallel the antigenic properties of the vaccine. It has been shown that vaccines of S pneumococci are equally good antigens whether heated at 60°C. or at 100°C.

It is possible that S vaccines may exert their effect in one of two ways:—(1) Directly on the R forms themselves; (2) On the tissues of the animals in which they are injected.

The failure of all *in vitro* attempts to secure transformation of type suggests that, if the vaccine exerts its influence directly on the living R forms, it does so only under very precise conditions. If it were possible to reproduce in the test tube the conditions obtaining in the subcutaneous tissues of the mouse, transformation of type might be effected *in vitro*. However, it would be difficult to duplicate experimentally the conditions created by the disintegration and digestion of large amounts of vaccine in the living tissues of an animal.

A second possibility is that the conditions created in the subcutaneous tissues of the mouse offer a suitable environment in which the R forms may build up their S structure. In the previous paper it was pointed out that, under natural conditions, the white mouse "possessed

some capacity to overcome infection by organisms producing minimal amounts of S substance." It was further suggested that "the injection of S vaccines might destroy or inhibit this limited ability of the mouse and under such conditions the R forms might develop into S organisms." May it not be possible that the injection of an S vaccine only inhibits or destroys the capacity of the mouse to overcome infection by that particular S type? Under such circumstances may not the R organism, potentially capable of synthesizing any type of polysaccharide, be able to elaborate that particular S substance most suitable for the survival of the organism in its environment?

In this connection reference is again made to the work of Sia (10). Employing serum-leucocyte mixtures in a specially constructed apparatus, he reported the following observation.

"The presence of a small amount of the purified soluble substance of the homologous type markedly altered the conditions in the mixtures so that even a small number of avirulent pneumococci were enabled to grow in the serum and leucocytes of animals which ordinarily possess the power to destroy such pneumococci in relatively large numbers." Sia further reported that this effect was highly type-specific for "a Type II substance assisted the growth of only pneumococcus Type II; likewise a Type III substance, the growth of pneumococcus Type III only."

Any such explanation, however, fails to account for the different effects produced by vaccines heated at temperatures above and below 80°C. Further work is therefore required to understand clearly the causes responsible for transformation of type as induced by Griffith's technique.

In the previous paper it was pointed out that R forms of pneumococcus could be found in the flora of the upper respiratory tract of many normal individuals. It was suggested that these forms resulted from attempts of the bacteria to adapt themselves to unfavorable environmental conditions. Although degraded to the R form these organisms still retained the capacity of again developing into virulent, type-specific, S pneumococci. Any such development would appear to be dictated by conditions in the environment. Those environmental conditions would also determine the particular S type which the R organisms may assume. In the absence of more precise data concerning such transformations further speculation is unprofitable. However, the possibilities of alteration in type under natural and

disease conditions cannot be ignored and may attain proportions of much significance in infectious and epidemiological problems.

## CONCLUSIONS

1. Type-specific S pneumococci may be transformed from one specific S type into other specific S types through the intermediate stage of the R form.

2. R forms of pneumococci, derived from any specific S type, may be transformed into S organisms of other specific types by the following procedure:—The subcutaneous injection, in white mice, of small amounts of living R forms together with vaccines of heterologous S cultures.

(i) S vaccines heated for 15′ at temperatures between 60° and 80°C., are effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(ii) S vaccines heated for 15′ at temperatures between 80° and 100°C., are not effective in causing R forms, derived from heterologous S types, to revert to the type of the vaccine.

(iii) S vaccines heated for 15′ at temperatures between 80° and 100°C., may cause 2 R and 3 R cultures to revert to their original S type.

(iv) S vaccines of any type, including Type I, heated for 15′ at temperatures between 80° and 100°C., are not effective in causing 1 R cultures to revert to their original S type.

(v) S vaccines heated for periods as long as two hours at 60°C. are effective in causing R forms, derived from heterologous types, to revert to the type of the vaccine.

3. A single cell R strain, derived from a Type II S pneumococcus, has been successively transformed into a Type III S, a Type I S and a Group IV S culture.

4. Corresponding with the various degrees of "degradation" of the R form there are varying degrees of "development" of the S form.

5. The nature of the conditions responsible for alteration of type as induced by these procedures has been investigated and the causes responsible for the transformations are discussed.

6. All attempts to produce transformation of type *in vitro* have been unsuccessful.

7. The rôle which the phenomenon of transformation of type may play in problems of infection and epidemiology is indicated.

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# SURVIVAL OF VACCINE VIRUS SEPARATED FROM LIVING HOST CELLS BY COLLODION MEMBRANES

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The fact that vaccine virus is able to multiply in the presence of living host cells is well established (1–5). None of the reports, however, regarding its increase in lifeless media has received general confirmation. Harde (6), using tissue cultures for the growth of vaccine virus, showed that the infectious agent was principally associated not with the plasma clot but with the cells. Parker and Nye (2) using an improved technique made observations similar to those of Harde. Although it is generally recognized that living host cells are essential for the *in vitro* multiplication of vaccine virus and are intimately associated with the active agent, no one definitely knows what function they serve in the cultures

For many years collodion membranes, in one form or another, have been used in bacteriology. Attempts have been made to study the relation of viruses to host cells by implanting collodion sacs containing the active agents in the peritoneal cavity of experimental animals. Williams and Flournoy (7) investigated vaccine virus in this manner and found that it survived only for a short time. Olitsky and McCartney (8) implanted the virus of typhus fever in the peritoneal cavity of guinea pigs and reported that it survived for 31 days. The objection to such experiments is that the collodion sacs with their contents acted as foreign bodies, so that, instead of the viruses being brought into close relation with normal host cells, they were brought into an indirect contact with fibrous capsule by which the bags became surrounded. This difficulty might have been avoided through the use of tissue cultures, were it not for the fact that in the past most of the techniques of such culture required the use of solid media in the form of plasma clots.

Recently Maitland and Maitland (9) reported that vaccine virus is capable of multiplication in a fluid medium consisting of a mixture of one third serum and two thirds Tyrode's solution in which bits of finely minced kidney tissue are suspended. These observations have been confirmed (10, 11). Such a fluid medium, in which living cells persist for at least 5 days (12), makes possible the use of semi-permeable membranes in investigations concerning the functions served by living host cells in the survival and multiplication of viruses.* In the present paper are reported observations on the survival of vaccine virus separated from living cells by collodion membranes.

## *Methods and Materials*

*Vaccine Virus.*—The neurovaccine virus of Levaditi was used. The testicles of rabbits, inoculated with a 1–10 dilution of the virus, were removed on the fourth day, emulsified in Locke's solution with sand, and centrifuged at high speed for 20 minutes. The supernatant fluid was used as an inoculum. The final dilution of the inoculum was identical in all of the preparations at the beginning of each experiment.

*Tests for Vaccine Virus.*—At the completion of each experiment, tests for the presence of active vaccine virus were made by emulsifying the different mixtures in a mortar without sand and injecting 0.25 cc. of each into the shaved skin of a rabbit.

*Dialyzing Apparatus.*—The dialyzing apparatus consisted of an outer tube flared slightly at the top to fit a shoulder on an inner tube, the two being ground to form a perfect joint (Text-fig. 1). The collodion sac was attached to the lower part of the inner tube by means of a rubber band. When 1 cc. of fluid was placed inside the collodion bag, and from 4 to 4.5 cc. in the outer tube, the fluid level was the same on the two sides of the membrane.

*Preparation of Collodion Sacs.*—The method of preparing the collodion bags is a slight modification of the one used by Northrop and Kunitz (13). Pyrex test tubes, with inside dimensions of 1.5 x 11 cm. were thoroughly cleansed and dried, after which 1.5 cc. of Merck's contractile collodion was placed in each tube. The tubes were then stoppered and allowed to stand until all bubbles had disappeared from the fluid, when the stoppers were removed and the tubes were placed horizontally in holders revolving at the rate of 20 revolutions per minute. After rotating for 5 minutes the tubes were removed from the machine and kept inverted for 2½ to 3 minutes. Then they were filled with water and the sacs were removed by permitting the fluid to run between the sacs and the walls of the tubes.

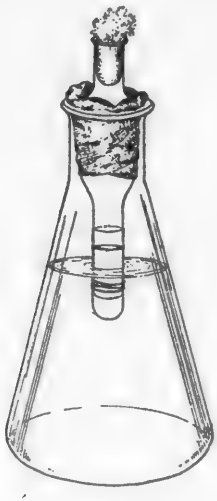
*Tests of Collodion Sacs.*—Each bag was cut to a length of about 6 cm. and the tip

* Semipermeable membranes have been used by Wollman, Bronfenbrenner, and Muckenfuss in investigations concerning the nature of bacteriophages (see J. Bronfenbrenner and R. Muckenfuss, *Jour. Exp. Med.*, 1927, **45**, 887).

of the inner tube of the dialyzing apparatus (Text-fig. 1) was slipped inside the sac until the disance from the end of the bag to the tip of the glass tube was 2.5 cm. The bag was then secured in place by a rubber band, the tube was completely filled with water, and a one-holed rubber stopper to facilitate connection with the testing apparatus described below was inserted in the top of the tube.

The testing apparatus consisted of a narrow glass tube about 1 meter in length at the lower end of which was a 2-way stopcock (Text-fig. 3). In one direction

TEXT-FIG. 1



TEXT-FIG. 2

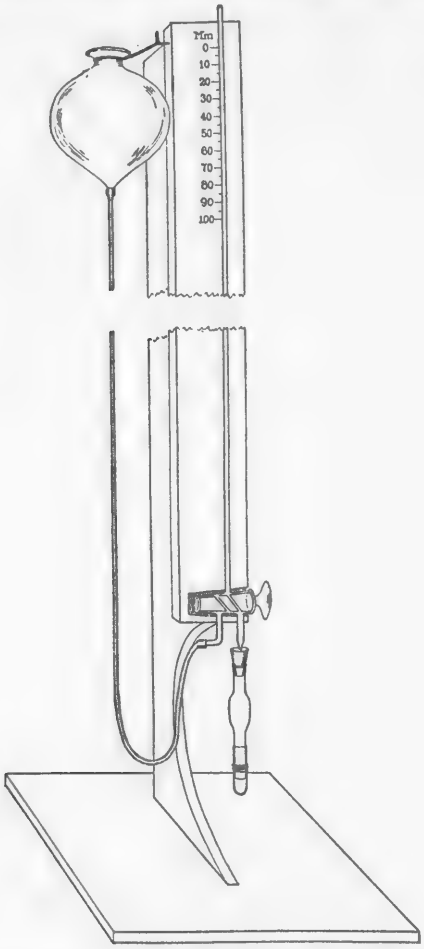
TEXT-FIG. 1. The inner and outer glass tubes of dialyzing apparatus. The collodion membrane fits over the lower part of inner tube and is held in place by an elastic band (see Text-fig. 2).

TEXT-FIG. 2. Manner in which inner tube with its collodion membrane is prepared for sterilization in the autoclave.

this led to a long rubber tube and a levelling bulb, which was filled with water. In the other direction it led to a straight glass tip which was drawn out to fit into the rubber stopper placed in the top of the inner tube of the dialyzing apparatus described above. The distance from the top of the collodion bag to the point on the support of the vertical glass tube, from which measurements were made, was 1 meter.

The apparatus was designed for the determination of the amount of water that would pass through the membrane in a given time under a pressure of 1 meter of water. The water level in the tube was adjusted to 1 meter, and the stopcock was

turned so that pressure was delivered inside the collodion sac. The bag was then examined for leaks and if none were present the amount of water passing through




TEXT-FIG. 3. Apparatus for testing the permeability of the collodion sacs.

in 3 minutes was read on the mm. scale beside the vertical tube. With the tubing used in our work a length of 9 cm. contained 1 cc. of water. Consequently, the

volume of water passing through the membrane was readily calculated. The membranes used permitted from 0.52 cc. to 0.78 cc. to pass through in 3 minutes.

*Sterilization of the Collodion Sacs.*—Upon completion of the tests, the tubes of the dialyzing apparatus were removed, part of the water was withdrawn, and the rubber stopper was replaced by nonabsorbent cotton. The shoulder of each inner tube was then wrapped with cotton strips surrounded by gauze and the tube itself was inserted into a 150 cc. Erlenmeyer flask, the cotton-gauze wrapping serving as a support (Text-fig. 2). The water level in the flask was above the top of the collodion bags but not as high as that inside the tubes. The flasks were then placed in the autoclave and sterilized under 5 pounds pressure for 45 minutes. The outer tubes of the dialyzing apparatus were sterilized by dry heat.

Several sacs were retested after sterilization and their permeability was found to be reduced to about one third of what it was before this treatment. The bags used in the experiments here reported were not again tested after sterilization.

*Maitland Medium.*—For the cultivation of vaccine virus, Maitland used chicken serum, Tyrode's solution, and minced chicken kidney in the following proportions: serum, 6 cc.; Tyrode's solution, 12 cc.; minced kidney, 0.66 cc. In the experiments reported in the present paper the Maitland technique was followed with the exception that rabbit serum and kidney were used.

## EXPERIMENTAL

For controls on the results of each experiment, data was obtained by testing the survival of vaccine virus alone in test tubes, and its persistence in the presence of living and frozen (last 2 sets of experiments) tissue both in test tubes and in Carrel flasks. For this reason some of tests were repeated more frequently than were others. In all, 24 experiments with collodion bags were performed. These are summarized in Table I which will be referred to from time to time as individual experiments are described.

As a preliminary study it was essential to ascertain whether vaccine virus would pass through the collodion sacs prepared in the manner above described, for, if it did diffuse through their walls, the bags would be unsuitable for the work.

### *Diffusibility of Vaccine Virus*

A freshly prepared testicular emulsion containing active vaccine virus was placed in the collodion sacs, and Locke's solution was placed in the outer chambers of 4 dialyzing apparatuses, 2 of which were placed in the incubator and 2 in the ice box. After 3 days the fluids on both sides of the sacs were tested for the presence of vaccine virus.

It was found that the virus retained its activity in the ice box and did not diffuse through the membranes. In the incubator the virus lost activity, consequently no data concerning its diffusibility at 37°C. was obtained. From the results of later experiments, however, it became obvious that vaccine virus will not diffuse through the type of membranes used.

The object of the first set of experiments was to determine if the survival of vaccine virus, suspended in a cell-free mixture of serum and Tyrode's solution, is enhanced by the presence of a culture of vaccine virus in Maitland's medium on the opposite side of a collodion membrane.

### *Survival of Vaccine Virus Separated from Living Cells Inoculated with Vaccine Virus*

In attempts to determine the effect that Maitland's medium inoculated with vaccine virus has on the survival of vaccine virus suspended in a cell-free mixture of serum and Tyrode's solution on the opposite side of a semi-permeable membrane, 6 experiments were performed as described below.

2 mixtures were prepared. One consisted of vaccine virus diluted 1–100 by a fluid made of one third rabbit serum and two thirds Tyrode's solution, the other was composed of vaccine virus (final dilution also 1–100) in Maitland's medium.* The suspension of vaccine virus was then placed on one side of the membranes in the dialyzing apparatuses, and Maitland cultures of vaccine virus on the other. The dialyzing sets were then placed in the incubator at 37°C. for 4 days, at the end of which time the fluids on both sides of the membranes were tested upon rabbits for the presence of active vaccine virus. As controls in each experiment virus (final dilution 1–100) was incubated in test tubes and in Carrel flasks.

The results of the above experiments with those to follow are summarized in Table I and show that vaccine virus alone survived in one third of the test tubes (controls), that it persisted or multiplied in Maitland's medium in all the dialyzing apparatuses, and that it survived in only one half of the dialyzing apparatuses when separated from the Maitland cultures by means of membranes. From these findings it is seen that the virus survived or actually multiplied,

* It must be remembered that Maitland's medium is prepared by mixing 0.66 cc. of minced normal kidney, 6 cc. of serum, and 12 cc. of Tyrode's solution.

as shown previously (10, 11), when it was in direct contact with living kidney cells, and that the survival of the virus in serum and Tyrode's solution was not influenced to any great extent when the active agent was separated from the Maitland cultures of the virus by means of semi-permeable membranes.

The possibility that the virus might have so severely injured the cells in the Maitland cultures that they were useless for the purposes of the experiment, or that the virus in the Maitland cultures required all of the factors necessary for survival, leaving none to diffuse through the membranes, was next considered. To test this point, uninoculated Maitland medium was placed on one side of the membranes, and vaccine virus, suspended in serum and Tyrode's solution, on the other.

*Survival of Virus Separated from Uninoculated Living Tissue*

12 experiments were made to ascertain what effect uninoculated Maitland medium placed in one chamber of the dialyzing apparatus had on the survival of vaccine virus suspended in serum and Tyrode's solution in the other chamber.

Vaccine virus was prepared and diluted with serum and Tyrode's solution as in the previous experiments, and Maitland medium was made in the usual manner. In the dialyzing apparatuses vaccine virus was then placed on one side of the membranes, and uninoculated Maitland medium on the other. After incubation for 4 days at 37°C. material from each side of the membranes was tested for the presence of vaccine virus.

The results of the experiments are summarized in Table I. Out of 12 tests the vaccine virus was found to survive in every case, and no virus was demonstrated in the Maitland medium on the opposite side of the membranes, except in one instance when virus passed through a leak in the bag. When the results of these experiments are examined and compared with those described above, it becomes evident that something from the living cells diffused through the membranes and supported the survival of the virus.

Although the virus survived in every instance, it seems that some of its activity was lost. In efforts to enhance the survival, it was next decided to run experiments similar to the ones just described, with the exception that dead cells (killed by repeated freezing and thawing)

TABLE I

*Summary of Experiments Concerning the Survival of Vaccine Virus at 37°C.*

| No. of tests | Containers | Contents | Results of tests for vaccine virus | |
|---|---|---|---|---|
| | | | Negative | Positive |
| 12 | Test tubes | Vaccine virus, serum, Tyrode's solution | 9 | 3 (+ to ++) |
| 12 | Test tubes | Vaccine virus in Maitland medium | 6 | 6 (± to +++) |
| 4 | Test tubes | Vaccine virus in Maitland medium (frozen) | 3 | 1 (+) |
| 12 | Carrel flasks | Vaccine virus in Maitland medium | 4 | 8 (+++ to ++++) |
| 2 | Carrel flasks | Vaccine virus in Maitland medium (frozen) | 0 | 2 (±) |
| 4 | Inner tube | Vaccine virus in Maitland medium | 0 | 4 (++++) |
| | Outer tube | Vaccine virus, serum, Tyrode's solution | 2 | 2 (+) |
| 2 | Inner tube | Vaccine virus, serum, Tyrode's solution | 1 | 1 (+) |
| | Outer tube | Vaccine virus in Maitland medium | 0 | 2 (++ to +++) |
| 6 | Inner tube | Uninoculated Maitland medium | 5 | 1 (leak in bag) |
| | Outer tube | Vaccine virus, serum, Tyrode's solution | 0 | 6 (+ to +++) |
| 6 | Inner tube | Vaccine virus, serum, Tyrode's solution | 0 | 6 (+ to ++) |
| | Outer tube | Uninoculated Maitland medium | 6 | 0 |
| 3 | Inner tube | Vaccine virus in frozen Maitland medium | 0 | 3 (+++ to ++++) |
| | Outer tube | Uninoculated Maitland medium | 3 | 0 |
| 3 | Inner tube | Uninoculated Maitland medium | 3 | 0 |
| | Outer tube | Vaccine virus in frozen Maitland medium | 0 | 3 (++++ to ++++++) |

Maitland medium consists of minced normal kidney suspended in serum and Tyrode's solution. Inner and outer tubes refer to the tubes of the dialyzing apparatuses. The pluses indicate the amount of vaccine virus present as determined by the severity of the reactions in the skin of rabbits.

were added to the suspensions of vaccine virus in serum and Tyrode's solution.

### *Survival of Virus in Contact with Dead Cells When Separated from Living Tissue by a Collodion Membrane*

A fresh emulsion of vaccine virus was prepared in the usual manner, care being taken to remove all cells by centrifugation. Fresh rabbit serum was collected from which all cells were removed. Fresh rabbit kidney was finely minced, half of which was frozen ($CO_2$ snow) and thawed 10 times.

A mixture of vaccine virus, serum, Tyrode's solution, and frozen kidney tissue was placed on one side of the membranes in the dialyzing apparatuses, and a preparation consisting of normal kidney tissue, serum, and Tyrode's solution on the other. The usual controls were set up. All preparations were incubated at 37°C. for 4 days, after which they were tested for the presence of active vaccine virus by intradermal inoculations (0.25 cc.) in rabbits. The results of the tests are given in detail below and are also summarized in Table I. The pluses indicate the amount of vaccine virus present as determined by the severity of the reaction in the rabbits.

| | Set A | Set B |
|---|---|---|
| Vaccine virus in test tubes | − | − |
| Vaccine virus, normal kidney, serum, and Tyrode's solution in test tubes | − | +± |
| Vaccine virus, frozen kidney, serum, and Tyrode's solution in test tubes | − | − |
| Vaccine virus, normal kidney, serum, and Tyrode's solution in Carrel flasks | ++++ | ++++ |
| Vaccine virus, frozen kidney, serum, and Tyrode's solution in Carrel flasks | ± | ± |
| Vaccine virus, serum, and Tyrode's solution in inner chamber of dialyzing apparatus | ++ | |
| Normal kidney, serum, and Tyrode's solution in outer chamber of dialyzing apparatus | − | |
| Vaccine virus, serum, and Tyrode's solution in outer chamber | +++ | |
| Normal kidney, serum, and Tyrode's solution in inner chamber | − | |
| Vaccine virus, frozen kidney, serum, and Tyrode's solution in inner chamber | +++ | ++++ |
| Normal kidney, serum, and Tyrode's solution in outer chamber | − | − |
| Vaccine virus, frozen kidney, serum, and Tyrode's solution in outer chamber | ++++++ | ++++++ |
| Normal kidney, serum, and Tyrode's solution in inner chamber | − | − |

The results of the experiments just described show (1) that vaccine virus in a mixture of dead cells (killed by freezing and thawing), serum, and Tyrode's solution lost its activity when incubated at 37°C. in test tubes for 4 days; (2) that vaccine virus, in a cell-free preparation of serum and Tyrode's solution placed in a dialyzing apparatus on the opposite side of the membrane from a mixture of normal cells, serum, and Tyrode's solution, retained some but not all of its activity; (3) that vaccine virus, in a mixture of dead cells (killed by freezing and thawing), serum, and Tyrode's solution placed in a dialyzing apparatus on the opposite side of the membrane from a preparation of normal cells, serum, and Tyrode's solution, apparently retained all of its activity. In the light of these findings, it seems that, under the conditions of the experiments, the living cells furnished for the survival of the virus something labile and capable of diffusing through semi-permeable membranes, while the cells killed by freezing and thawing supplied another factor relatively stabile and non-diffusible. However, this explanation of the observed phenomena should not be accepted as correct until it is substantiated by further work.

## DISCUSSION

Maitland's fluid medium and a simple dialyzing apparatus devised by us made possible investigations concerning certain functions served by living host cells in the survival of vaccine virus at 37°C. Since collodion sacs, through which vaccine virus could not diffuse, were used in the dialyzing apparatuses, it is obvious that the prolonged survival of the virus on one side of the membranes was not dependent upon a direct contact with the living host cells on the opposite side. Therefore, it appears that the living cells furnished something capable of reaching the vaccine virus by diffusion. Inasmuch as a number of substances, even gases in solution (14), can pass through the type of membranes employed, the nature of the diffusible substance supplied by the living cells is not yet known. Experiments, designed to obtain more information concerning the phenomena described in this paper, are now being conducted and the results of these investigations will be reported later.

SUMMARY

Vaccine virus, suspended in a mixture of serum and Tyrode's solution and separated by collodion membranes from a suspension of living kidney cells in serum and Tyrode's solution, remained active at 37°C. for a longer period of time than did vaccine virus incubated only in a mixture of serum and Tyrode solution.

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# A COMPARISON OF THE METHOD OF EXCRETION OF NEUTRAL RED AND PHENOL RED BY THE MAMMALIAN KIDNEY*

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During the last decade a considerable addition to our knowledge of kidney function has been made by the study of renal activity in the comparatively simple mesonephroi of the amphibia. The kidney of the frog in particular has been subjected to investigation by a long series of observers who have used methods, such as direct observation (Richards (1)) and perfusion (Höber (2)), to which the mammalian kidney does not readily lend itself. As a result facts have been ascertained directly concerning processes which up to the present time have been investigated in mammals only by indirect methods. These latter methods have of necessity been based largely on hypothesis and have produced chiefly theory, so that if the phenomena observed directly in the frog's kidney could be demonstrated in the mammalian kidney a considerable advance would be made. It has been tacitly assumed by many that an analogy between the two types of animals is a proper one, but since little if any direct evidence has been produced to support such a contention the present study is offered as an attempt at such experimental confirmation.

The possibility of the investigation arose in the following way. In a recent study of the manner of excretion of phenol red and neutral red by the perfused frog's kidney an interesting contrast was found in the mechanism of elimination of the two dyes (Oliver and Shevky (3)). Phenol red was found to be excreted chiefly through the glomeruli

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while the more colloidal neutral red was eliminated almost entirely through the tubules. It would seem, therefore, that with such a striking dissimilarity in the manner of excretion of these two dyes by the frog's mesonephroi that experiments on the excretion of the two dyes by mammals, under varying conditions, might cast some light on the question as to the possibility of analogous contrasting mechanisms of elimination in mammalian kidneys.

Another point of interest also presents itself in the study of the excretion of the two dyes by mammals. Phenol red has long been used as a clinical test of kidney function. If it should prove to be the case that neutral red is excreted in a different manner and by a different mechanism by mammals it is possible that it might serve as an adjunct to the phenol red test of the kidney.

With these points in mind the excretion of the two dyes has been studied under various conditions in frogs and rabbits.

### *Frog Experiments*

*Methods.*—In the frog experiments the following methods were used. The kidneys of large Rana catesbiana of from 850 to 1000 gms., weight were perfused by the method which we have previously described in detail (3). Very briefly, the perfusion fluid is a modified Locke's solution containing sugar which is led to the kidneys by the renal arteries, thus supplying the glomeruli and at the same time by the renal portal venous system which perfuses the tubular circulation. Pressures of 40 and 20 cm. of water are used on the artery and vein respectively.

Under these conditions the kidney produces a urine which is normal in amount and in its constituents. The volume varies from 7 to 10 ccm. per hour, urea is concentrated, salts are diluted, sugar is retained and colloidal substances such as proteins and gum arabic do not pass into the urine. The two dyes phenol red and neutral red, if added to the perfusion fluid in concentrations of around 15 mgs. per 1000 ccm. of fluid, are concentrated many times and as we have stated are excreted by the glomeruli and tubules respectively.

The analytical methods used in studying the urine were as follows: For sugar, Benedict's qualitative solution, for the dyes a Duboscq colorimeter and for the salts a Christiansen ionometer, the results of this latter determination being expressed as per cents of NaCl.

*Experimental.*—In figure 1 is shown the experimental results of perfusing the frog's kidney with a Locke's solution containing urea and phenol red in concentrations of 20 mg. and 500 mg. per liter re-

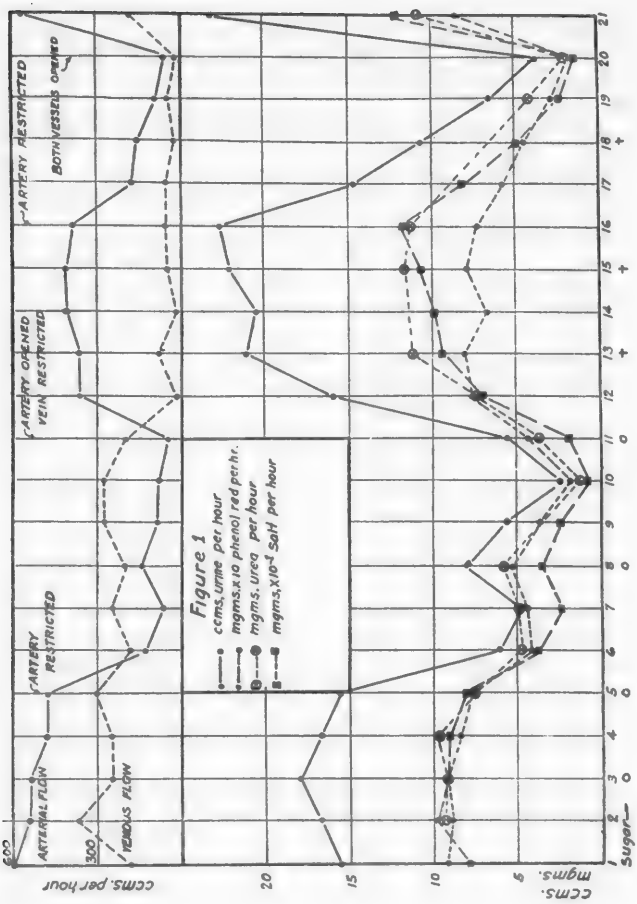
ARTERY RESTRICTED
BOTH VESSELS OPENED
ARTERY OPENED
VEIN RESTRICTED
ARTERY RESTRICTED
ARTERIAL FLOW
VENOUS FLOW
ccms. per hour
Figure 1
ccms. urine per hour
mgms. x 10 phenol red per hr.
mgms. urea per hour
mgms. x 10⁻⁴ salt per hour
ccms.
mgms.
Sugar

FIG. 1

spectively. These substances were present in the fluid which supplied both the glomeruli and the tubules, so that they were available for excretion by both of these elements of the kidney. In the upper portion of the chart is seen the outflow for the two circulatory systems after the fluid had passed through the kidney.

For the first five periods the perfusion was allowed to proceed in a normal manner. The volume of urine obtained was somewhat higher than that usually obtained, averaging about 16 ccm. per hour but the amount was fairly constant during the entire five periods. No sugar was present in the urine. The concentration ratio, i.e., the concentration of the phenol red in the urine as compared to the concentration in the perfusion fluid was also fairly constant, averaging from a 250 to a 300 per cent increase. The rates of excretion, expressed as mgs. per hour, of urea phenol red and salt were also constant. They averaged 10 mgs. per hour for urea, 1 mg. per hour for phenol red and 100 mgs. per hour for salts.

At the end of the fifth period the flow through the glomeruli was restricted by clamping the rubber tube which leads to the arterial cannula. This resulted in a marked decrease in the arterial outflow, while the circulation through the venous tubular system remained unaffected. Very marked changes were noted at once in the urine. The volume decreased to 6 ccm. per hour, a fall of 62.5 per cent. The rates of excretion of urea, phenol red and salt also fell to approximately one-half their former value.

These conditions continued through the next five periods, during which time the glomerular circulation remained low. The venous circulation remained adequate, however, for at no time was there sugar in the urine.

The interpretation of these findings in the light of our previous findings (3) is plain. A lessened supply of urea, salt, phenol red and water to the glomeruli produced a corresponding decrease in the rate of excretion of these substances and this in spite of the fact that these substances were being administered in excess to tubules which, judged by their ability to absorb sugar, were entirely normal. The major source of excretion of all these substances must have been the same therefore, and this source the glomeruli.

The converse experiment was now performed. At period 11 the

glomerular supply was increased by removing the constriction on the artery and the venous supply to the tubules decreased by restricting the flow to the vein. There was an immediate reestablishment of the former levels of excretion of water, salt, phenol red and urea. In the 13th period the effect of the tubular insufficiency became evident for sugar appeared in the urine, a sign of lack of tubular absorption. For this same reason a moderate "tubular diuresis" resulted, for it will be seen that in periods 13 to 17 although the arterial flow through the glomeruli is less than during the periods of normal perfusion (1–5), nevertheless the rate of water excretion is considerably higher. The same is true of the rates of excretion of urea and salts. In the case of phenol red the previous normal level was not exceeded in this particular experiment.

Under the conditions of this phase of the experiment, therefore, that is with adequate glomeruli supply and with frankly damaged tubules, a result of "anemia" and lack of oxygen, the kidney excreted phenol red, urea and salts at a rate equal to that of the normal kidney. The latter two substances were even excreted at an increased rate, since these substances as has been shown by other methods, are absorbed from the lumen of the tubule when the tubular epithelium is functioning normally.

The remainder of the experiment repeats the previous demonstration of the effect of glomerular "anemia." It will be seen that the rates of excretion of urea, salt and phenol red vary together and follow the rate of excretion of water, and that all these rates depend on an adequate glomerular supply. In contrast to the predominantly glomerular excretion of phenol red figure 2 shows a similar experiment in which the kidneys were perfused with Locke's solution containing urea and neutral red in concentrations of 12.5 and 500 mg. per liter. As in the first experiment both urea and dye were administered to tubules and glomeruli simultaneously by artery and vein and were therefore available for excretion by both of these parts of the renal unit.

In the first period 15 ccm. per hour of urine was excreted. The rate of urea excretion was 15.9 mg. per hour and the rate of neutral red excretion 4.3 mg. per hour. Salts were eliminated at the rate of 48 mgs. per hour. The concentration ratios of the various substances

have not been charted as their variations throughout the experiment are of much less significance than the rates of excretion. During the first period the dye was 23 times as concentrated as the perfusion fluid and the urea three times as concentrated.

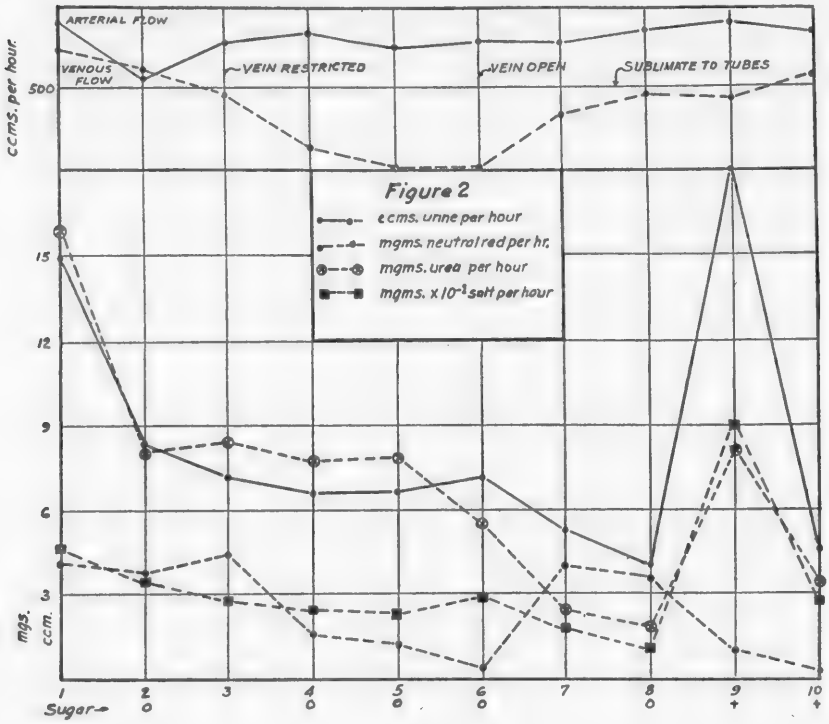


FIG. 2

In period 2 the arterial flow through the glomeruli was lessened and, as a result, the volume of urine excreted fell to 8.6 ccm. per hour. The rate of urea excretion followed this drop to 8.4 mg. per hour, as did the rate of salt excretion (34 mg. per hour), these results being similar in nature to those of the previous experiment. A striking difference is noted in the rate of excretion of the dye. Instead of falling, as did the rate of phenol red excretion in the first experiment,

its rate of excretion was unaffected by the change in glomerular elimination as it remained approximately equal to the previous period. Period 3, under the same conditions, was essentially the same as period 2.

At this point the supply of dye to the tubules was decreased by restricting the flow of perfusion through the vein. In this experiment, however, no actual damage to the tubule cells was produced. No diuresis developed nor did sugar appear in the urine as a result of lack of tubular absorption. The glomerular circulation remained as in the previous periods and the rate of water, urea and salt excretion continued through the next three periods (4, 5 and 6) essentially unchanged. But again the striking contrast in the excretion of the two dyes is seen, as the rate of neutral red elimination instead of following that of urea as did phenol red in the previous experiment, fell progressively to a final figure of .56 mg. per hour.

At the end of period 6 the vein was reopened and the tubules supplied with the former amount of neutral red. Since the tubules, as just noted, had been undamaged in this experiment the rate of neutral red excretion immediately rose to its former figure, 4.1 mg. per hour.

At the end of period 8 a new procedure was introduced into the experiment. 10 ccm. of $\frac{1}{4000}$ corrosive sublimate was administrated to the tubules during a 10 minute interval at low pressure. We have described elsewhere the result of such a procedure (3). It is followed by all the results of tubule damage, i.e., diuresis and escape of sugar into the urine and, as we will show in a later study, is frequently followed by a repression of urine, probably the result of vascular damage. Our interest here is only in how the dye excretion varies under these unusual conditions.

As will be seen in period 9 a diuresis developed, 18 ccm. per hour, and large amounts of sugar appeared in the urine. As in the previous experiment when the tubules were damaged by "anemia," the rate of urea excretion increased, as did that of salt elimination. But the rate of neutral red excretion instead of accompanying this rise as did phenol red in the first experiment, fell at once and finally reached the extremely low figure of .07 mg. per hour.

In periods 10 and 11 the typical sublimate repression of urine developed and with the fall in water excretion there went the usual fall in rates of urea and salt output.

These two experiments illustrate again the antithesis in the excretion of the two dyes. One, phenol red, behaves as if it were excreted principally by the glomeruli; the other, neutral red, as if the tubules were its chief source of elimination. But for the experiments with mammals which are to follow, the important contribution of these experiments is that the rate of urea excretion may be used as a standard to which the dye excretions may be compared.* It is by taking advantage of this fact that the following study of the methods of excretion of the two dyes by the mammalian kidney is made possible.

### *Mammalian Experiments*

In following the elimination of dyes and urea by the perfused frog's kidney only the actual rate of excretion of these substances need be followed, for one of the most important factors in determining this rate, namely the concentration in the perfusion fluid, is constant. In the experiments on living rabbits designed to determine whether or not the methods of excretion shown to exist in the amphibian kidney can be transferred to the mammalian kidney, the maintenance of such a state is impossible, since the perfusion fluid here is the plasma of the circulating blood. Experience with the excretion of other substances, such as chloride (4), phosphate (5, 6), creatinine (7, 8) and particularly urea (9), has shown that in the absence of definite knowledge of the behavior of a substance that not the rate of excretion alone but this rate in relation to the plasma concentration or the excretory ratio: $\frac{\text{Urine rate}}{\text{Plasma concentration}}$ should form the basis of study and comparison. In the choice of conditions under which to observe this ratio experience gained in the study of urea excretion was again called upon. The rate of urea excretion by the kidney is influenced by a variety of factors other than the blood urea concentration. Constancy of these factors is best obtained (10) when there is a marked stimulation of renal tissue or when as Addis has pointed out (11) the

* We do not wish to discuss at this time the mechanism of urea excretion, reserving this for a later communication. In these experiments its method of excretion resembles that of phenol red and its chief source of elimination is the glomerulus.

conditions are such that it seems reasonable to assume that all of the renal elements have been wakened to activity. It seemed highly probable that other factors than the dye concentration of the plasma may accelerate or inhibit phenol red and neutral red excretion and, in that case, it is to be expected that a balance of these factors would also best be obtained during a heightened renal activity. This was attained by the administration of urea and large quantities of water. During the ensuing diuresis simultaneous observations of the serum dye concentration and the rate of dye excretion in the urine were made.

*Methods.*—Healthy male rabbits were chosen for these experiments. When observations were terminated the animals were killed with ether and whenever the kidneys were not entirely normal the experiment was discarded. Except for the low urine volume experiments the general procedure was identical for every animal. No food was given for fifteen hours before the experiment commenced. Three hours before the first catheterization, when observations were begun, 40 ccm. per kilo body weight of a 5 per cent solution of urea was given by stomach tube and every hour thereafter until the experiment was ended 40 ccm. of tap water per kilo was administered in the same manner. The urine was obtained by catheter at approximate intervals as recorded in each experiment. After each catheterization the bladder was thoroughly washed with a known volume of distilled water. Arterial blood was obtained from the heart without an anticoagulant at the middle of each period of urine collection. Analyses were performed on the serum. There is scarcely a demonstrable difference in the urea or dye concentration of serum and paraffine plasma from the same sample of blood. Urea in both the serum and urine was determined by a urease and aeration method. Phenol red and neutral red in the serum and plasma were determined by the addition of NaOH in one case and HCL in the other and comparison after the necessary dilution with known standards in a Dubosq colorimeter. The results are all expressed as milligrams excreted per hour and milligrams per 100 ccm. of serum.

*Comparison of the Excretion of Phenol Red and Neutral Red*

Numerous experiments were performed with varying degrees of success in comparing the excretion of phenol red with neutral red. The phenol red experiments were usually successful while with the more colloidal neutral red difficulties were encountered. At the normal pH of rabbit plasma this dye in comparison with phenol red is relatively insoluble and in attempting to obtain higher and higher plasma concentrations some animals were killed in so-called "ana-

phylactoid" shock. Whatever the quantity of dye injected by far the greater part rapidly leaves the blood stream and as compared with phenol red only relatively low plasma concentrations can be

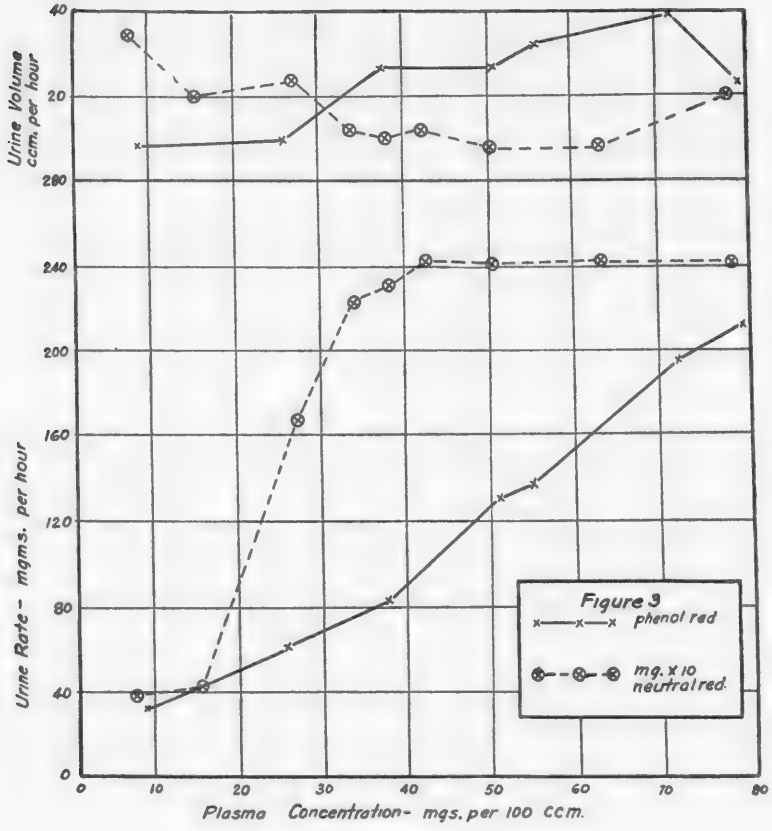
Urine Volume c.cm. per hour
Urine Rate - mgms. per hour
Plasma Concentration - mgs. per 100 c.c.m.
Figure 3
phenol red
mg. x 10 neutral red.

FIG. 3

obtained. Three or more observations were obtained for both dyes in 14 rabbits. In every case the main results were the same. A typical experiment follows:

A male rabbit weighing 2500 grams was given water and urea as described and 3 hours later 500 mgms. of phenol red were given intravenously in 2 per cent

solution. Ten minutes after this the bladder was washed out and the first urine collection commenced. Urine was collected at half hour intervals and serum specimens obtained at the middle of these periods. The observations are plotted in figure 3. Six days later this rabbit was again given urea and water. Thirty minutes before the first urine collection was commenced 100 mgms. of neutral red in 1 per cent solution were injected intravenously. Fifty mgms. of the dye were given every 30 minutes thereafter until the end of the experiment. Urine collections were made at half hour intervals and serum specimens obtained as before.

In figure 3 the neutral red observations have been compared with the phenol red excretion figures. There can be little question that neutral red is excreted in a manner very different from that of phenol red by the mammalian as well as the amphibian kidney. The rate of phenol red excretion in the urine is directly proportional to the plasma dye concentration while the rate of neutral red excretion bears no constant relation to the concentration of neutral red in the plasma except that a maximum rate of excretion independent of the plasma dye concentration is reached while the latter is still at a low figure.

An experiment reported by Marshall and Crane (12) which with other data led these authors to conclude that phenol red was secreted by the tubules of both the amphibian and mammalian kidneys requires some comment at this point. In comparing the rate of phenol red excretion to the plasma phenol red concentration in a dog these investigators failed to find the direct relationship which we have described but instead obtained a curve resembling our own neutral red observations. An examination of the protocol of their experiment reveals possible reasons for the discrepancy between their results and ours. They collected urine specimens as small as 1.0 cc. and even with the ureters cannulated these volumes are too small to give any degree of accuracy to urine collections from a dog's kidney when the tubule and pelvis dead space is taken into consideration. Another factor which would nullify their unsupported observations is the time interval used for urine collections. In no instance did this exceed the very short period of 3 minutes. Another and perhaps the most important factor contributing to the nature of their results is the fact that blood samples were taken not at the middle but at the beginning of the 3 minute periods of urine collection. Presumably this was done to correct for the urine volume of the kidney dead space and would conceivably have done this had the urine volume remained the same throughout the observations. This however was not the case and the deviations from a direct plasma concentration—urine rate relationships are in a general way those which might be expected from the variation in the urine volume.

*Comparison of the Appearance Time of Phenol Red and Neutral Red*

Another marked difference in the excretion of phenol red and neutral red which is not shown in the type of experiment illustrated in figure 3 is the interval between the commencement of the intravenous injection of the dye solution and the time of its appearance in the bladder urine. In comparison with phenol red neutral red was always very slow in making its appearance in the urine. By continuous washing of the bladder during a marked diuresis the dye appearance time was determined with a fair degree of accuracy in a number of experiments. In the experiments where it was recorded neutral red appeared in the bladder urine 3, 5, 3, 9, 12, 15, 16, 16, 17 and 22 minutes after the intravenous injection was begun. The appearance time apparently bore no relation to the dose. In one case phenol red required over 4 minutes to reach the urine but in more than a dozen other experiments the appearance time was uniformly less than 1 minute. If we assume an analogy between the excretory mechanisms of the frog and rabbit kidneys in so far as these dyes are concerned it is easy to visualize the reason for the marked difference in the appearance time of these two dyes. Phenol red would pass through in the glomerular filtrate almost immediately on reaching a *fully active* kidney and would soon be seen in the urine. On the other hand it would seem reasonable that neutral red, if secreted by the tubules, requires a measurable length of time to be removed from the plasma into the cells and passed into the urine in any amount.

*Comparison of Phenol Red and Urea Excretion*

Phenol red and urea are excreted in a similar manner by the perfused frog's kidney. Proof that this was also true in the rabbit would contribute additional evidence indicating an analogy in the mechanisms of renal function of these two types of animals. The following experiment is typical of a group of five in which excretion of phenol red and urea were simultaneously compared.

A male rabbit weighing 2.5 kilos was treated as previously described and 15 minutes before the collection of urine was begun the animal received 15 cc. of 5 per cent phenol red solution intravenously. Urine was collected over eight

30-minute periods and blood samples taken at the mid-point of each of these periods. The results form figure 4.

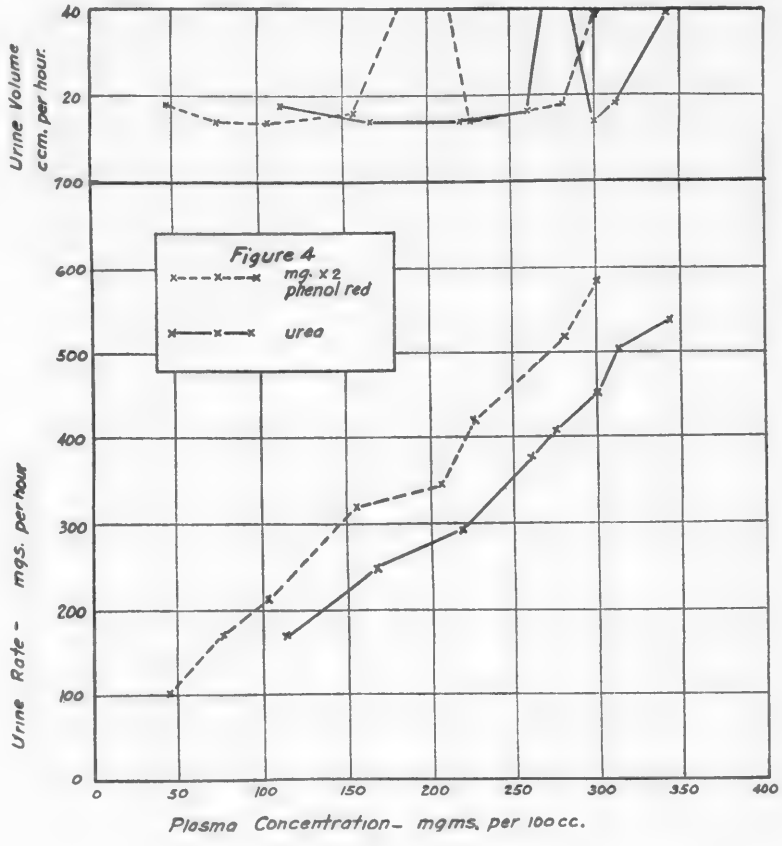


FIG. 4

Phenol red then is excreted by the mammalian kidney in the same way as urea, that is, in both cases the rate of excretion in the urine is directly proportional to the plasma concentration. In the particular experiment cited here the ratios: $\frac{\text{Urine Rate}}{\text{Plasma Concentration}}$ are rather small for both urea and phenol red in relation to the size of the rabbit

and at postmortem the kidneys were found to be normal but considerably smaller than usual. The difference between the ratios, that of phenol red being higher than that of urea, is a constant finding which will be discussed in another communication.

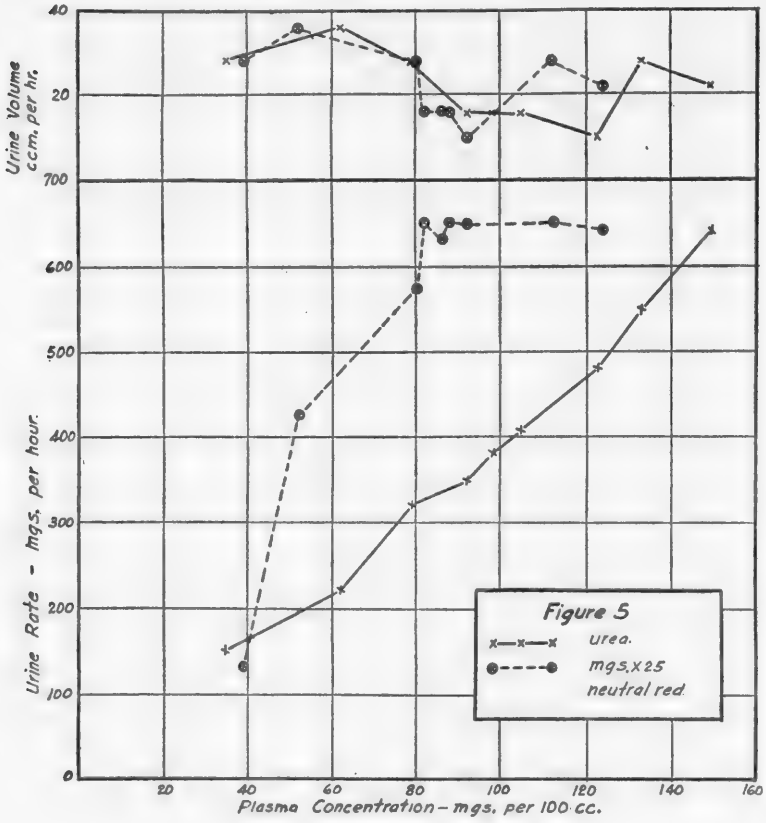


FIG. 5

*Comparison of Neutral Red and Urea Excretion*

In contradistinction to phenol red, neutral red is excreted by the amphibian kidney in a manner unlike urea. Likewise neutral red

and urea are excreted differently by the mammalian kidney. A typical experiment, one of three in which this point is demonstrated, follows:

A 3.5 kilo male rabbit received water and urea as described previously. Neutral red was given intravenously as in experiment 3, 175 mgms. being injected in 1 per cent solution each half hour. Nine 30-minute urine collections were made, a sample of serum being obtained at the middle of each one.

The results in figure 5 show the usual relationship under these conditions for urea, the urine rate being directly proportional to the plasma concentration. The excretion of neutral red on the other hand bears no consistent relation to the plasma concentration at low levels of the latter and at higher levels the rate remains constant despite further increases in the concentration of dye in the plasma.

*Relation of Phenol Red Excretion to Urine Volume*

Rowntree and Geraghty (13) and other observers (Marshall and Kolls (14)) have held that the rate of phenol red elimination by the kidneys is independent of the fluid output. If this were so the excretion of this dye would be different in this respect from urea. Although no direct relationship between the rate of urea excretion and the urine volume has been demonstrated it has been shown (15, 16) that both the rate of urea excretion as measured by the ratio: $\frac{\text{Urine Urea Rate}}{\text{Blood Urea Concentration}}$ and the urine volume tend to vary with the degree of renal activity. Low urine volumes and low urea ratios are generally found at low degrees of renal activity and increase with the degree of renal activity until the urea ratio is at a maximum when the urine volume alone may continue to increase. If phenol red and urea are excreted by the same mechanism the relationship of the excretory ratios to the urine volume should be similar. That this is so in the frog's kidney has been shown in the experiments already described and the experiment which forms figure 6 demonstrates that this is also the case in the mammalian kidney. These observations were obtained as follows:

A 3.5 kilo male rabbit was kept without food or water for 15 hours. At the end of this period 3 ccm. of a 5 per cent phenol red solution were injected intra-

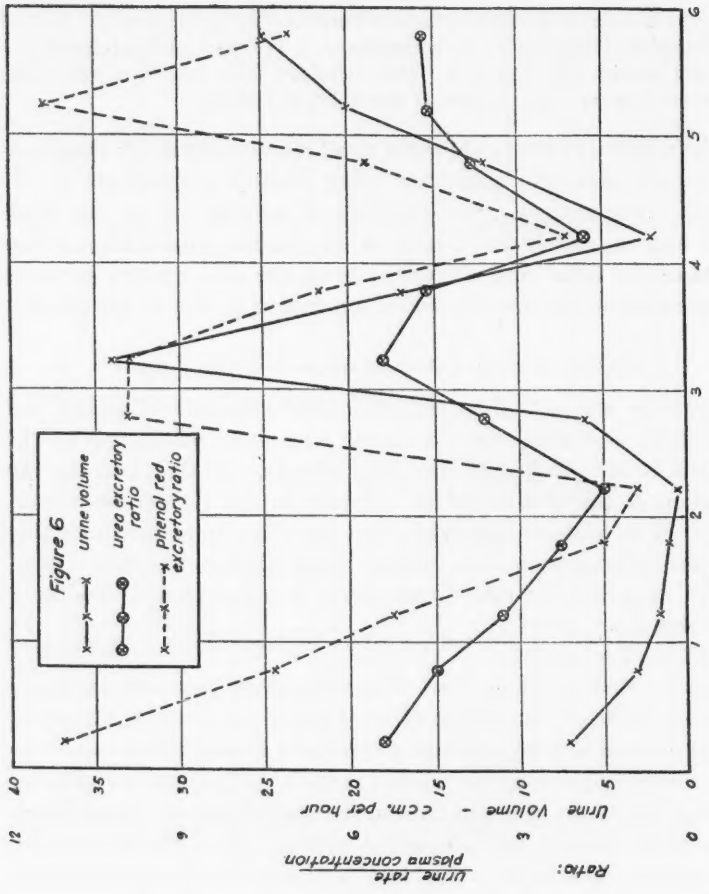
Figure 6
urine volume
urea excretory ratio
phenol red excretory ratio
Time in hours
Urine Volume - c.c.m. per hour
Ratio: urine rate / plasma concentration

FIG. 6

venously and ten minutes later the bladder was drained and washed with several small known volumes of distilled water all of which was returned. During the ensuing 6 hours 12 half-hour urine collections were made, the bladder being washed out at the end of each one with four 10 cc. portions of water. A sample of blood was obtained at the middle of each urine period. One and a half hours after the first urine collection was commenced 3 cc. more of 5 per cent phenol red solution were given by ear vein. At 2.5 hours 100 cc. of distilled water were given by mouth. Five cc. of 2 per cent urea and 2 per cent phenol red were given intravenously at 3 hours and 10 cc. at 4.5 hours. At 5 hours the rabbit received 10 cc. of 20 per cent creatinine and at 5.5 hours 15 cc. of 20 per cent creatinine and 0.2 per cent phenol red by intravenous injection.

The observations in this experiment show very clearly that the rate of phenol red excretion, just as does the rate of urea excretion, fluctuates with the urine volume under conditions of less than full renal activity. This result is at variance with the view which is generally held that no changes in urine volume are associated with changes in phenol red excretion. The reason for this is that previous observers have neglected to take into consideration the plasma dye concentration and have dealt not with the rate of excretion but with the percentage of an injected quantity which appeared in the urine in an arbitrary period.

## SUMMARY

A direct examination with the method of perfusion of the excretion by the frog's kidney of phenol red and neutral red has shown that the dyes are eliminated in different manners as a result of different mechanisms. The former is excreted in much the greater part by the glomeruli; the latter by the tubules. Urea is excreted in a manner similar to phenol red.

The indirect examination of the function of the mammalian kidney by means of excretion ratios has shown a like contrast between the manner of elimination of the two dyes, and here again was found a similarity in the manner of excretion of phenol red and urea.

This would seem to be as close an examination as can be made with our present methods of experimentation of the question of the mechanism of the excretion of these substances by mammals. As the facts stand they constitute strong presumptive evidence that in mammals and amphibia the like results have arisen from like causes,

phenol red and urea being eliminated chiefly through the glomeruli in both instances while neutral red is excreted principally through the tubules.

## CONCLUSIONS

1. There is a difference in the manner of excretion of phenol red and urea from that of neutral red by the frog's kidney.
2. This difference is due to differences in mechanism of excretion, the elimination of the former being by the glomeruli and the latter by the tubules.
3. There is a similar difference in the manner of excretion of these substances by the mammalian kidney.
4. It is inferred that this similar difference in manner of excretion is due to a similar difference in method of elimination, and that in mammals too, phenol red is eliminated chiefly through the glomeruli and neutral red through the tubules.

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